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(54) Title: POLYPEPTIDE DOMAINS CAPABLE OF BINDING RHO PROTEIN AND THEIR USE IN ASSAYS		
(57) Abstract <p>The Rho family of GTPases are involved in a wide range of eukaryotic cellular processes. Several known effector proteins bind to these GTPases and mediate their effect. Such proteins include ROCK-I/p160ROCK, ROCK II and Kinectin. The present invention provides a novel twenty three amino acid Rho-binding region which is conserved in a range of Rho binding proteins. Other proteins, including the yeast protein Skn7, are shown to contain regions homologous to this conserved region and to interact with Rho GTPases. Assays are also provided to identify molecules which affect cell growth through modulation of the Rho GTPase/effector protein interaction.</p>		

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POLYPEPTIDE DOMAINS CAPABLE OF BINDING RHO PROTEIN AND THEIR
USE IN ASSAYS

Field of the Invention.

- 5 The present invention relates to the Rho family of GTPases and their interactions with effector proteins in eukaryotic cells, particularly mammalian and yeast cells.

Background to the Invention.

- 10 RhoA is involved in a number of cellular processes, including stress fibre formation, cell motility, cytokinesis, control of proliferation and apoptosis. It exerts its effects through binding to a number of effector proteins, a number of which have been characterised. These effector proteins include
15 ROCK-I/p160ROCK, and ROCK-II, and their rodent homologues, Kinectin, p140mDia and mDia2 (mammalian homologues of *Drosophila* Diaphanous), Rhoophilin, Rhotekin, Citron, and the PRK protein kinase family.
- 20 Some of the proteins which interact with RhoA are relatively large, and it is of interest to determine regions within these proteins responsible for the RhoA interaction. The identification of specific regions would facilitate understanding of the biology of the proteins, and assist in
25 the development of targets suitable for assays for the identification of candidate modulators, particularly inhibitors, of the interaction. Rho proteins appear to act in subtly different ways with their various effector proteins, and these various interactions appear to give rise
30 to the diversity of effects mentioned above. Better understanding of the mechanisms of interactions will allow rational drug design which target these interactions, and particularly provide means of selectively targeting specific subsets of interactions.
- 35 Leung et al (Mol. Cell. Biol. (1996) 16; 5313-5327) describe the interaction of ROK α with RhoA. ROK α is a protein with

serine/threonine kinase activity which plays a role in the restructuring of the cytoskeleton. The authors pinpointed a stretch of 20-30 amino acids, found within amino acids 893-982 of the protein, which was reported to be responsible for the RhoA interaction. This corresponds to residues 949-1039 of ROCK-I. Fujisawa et al (J. Biol. Chem., (1996) 271, 23022-23028) describe the identification of the Rho-binding domain of ROCK-I and reports that the region 934-1015 is the sole RhoA binding domain. Both the rat and human regions contain a highly conserved stretch of amino acids at 995-1013 and Fujisawa et al, *ibid*, report that mutations at positions 1008 and 1009 significantly reduced and abolished RhoA binding respectively. Additional mutations at positions 934 and 941 were also reported to significantly attenuate the signal.

Hotta et al, (Biochem. Biophys. Res. Commun. (1996), 225; 69-74) report the interaction of RhoA with kinectin, a vesicle membrane anchoring protein of a kinesin ATPase motor. The authors identified residues 630-935 of human kinectin as containing a RhoA binding domain.

Disclosure of the Invention.

We have investigated the interaction of RhoA with effector proteins using both yeast two-hybrid and *in vitro* biochemical assays. Under our assay conditions we have surprisingly found that the highly conserved ROK/ROCK region is not necessary for RhoA binding and instead the binding is mediated by a less conserved region found at residues 949-972 of ROCK-I. This is C-terminal to the residues 934 and 941 and N-terminal to the residues 1008 and 1009 identified in the prior art to be of significance. We have further found that the 23 amino acid sequence we have identified is conserved in Kinectin at residues 1092-1115, outside the previously identified binding domain, although a less well conserved sequence homology is found at residues 831-854. The 1092-1115 region of sequence homology is within the

region of residues 1053 to 1327 which we have identified as the Rho-interacting domain of kinectin.

We have further found that this region is conserved in other proteins. One of these other proteins is Skn7, a yeast protein which is involved in DNA binding and which is required for yeast in response to oxidative stress. Another is heterotrimeric G protein β subunit ($\beta 2$). Neither of these proteins have previously been identified as Rho binding proteins although the present work indicates that both can interact with both human RhoA and its yeast homologue Rho1.

The present invention thus provides an assay for a putative modulator of cell growth comprising:

- a) bringing into contact a Skn7 polypeptide, a Rho polypeptide and a putative modulator compound under conditions where the Skn7 polypeptide, in the absence of modulator, is capable of binding to the Rho polypeptide; and
- b) measuring the degree of modulation of binding between the Skn7 and Rho polypeptides caused by said modulator compound.

In a further aspect, the invention provides an assay for a putative modulator of cell growth comprising:

- a) bringing into contact a $\beta 2$ polypeptide, a Rho polypeptide and a putative modulator compound under conditions where the $\beta 2$ polypeptide, in the absence of modulator, is capable of binding to the Rho polypeptide; and
- b) measuring the degree of modulation of binding between the $\beta 2$ and Rho polypeptides caused by said modulator compound.

In another aspect, the invention provides an assay for a putative modulator of cell growth comprising:

- a) bringing into contact a polypeptide consisting

- essentially of a ROCK/Kinectin homology domain, a Rho polypeptide and a putative modulator compound under conditions where the homology domain polypeptide, in the absence of modulator, is capable of binding to the Rho polypeptide; and
- 5 b) measuring the degree of modulation of binding between the homology domain and Rho polypeptides caused by said modulator compound.

10 In a further aspect, the invention provides a polypeptide in isolated form which consists essentially of a ROCK/Kinectin homology domain, or a polypeptide in isolated form comprising said domain linked at the N- or C-terminus to an amino acid sequence not naturally found linked to said domain.

15 The invention further provides nucleic acid vectors encoding the domain polypeptides of the invention, means for their expression and means to recover such polypeptides as a result of said expression. The polypeptide may be used as potential modulator compounds in assays of the invention. The domain polypeptides may be formulated in the form of a pharmaceutical composition containing one or more of said polypeptides with a suitable carrier or diluent.

25 Description of Drawings

Figure 1 shows the sequence of the RhoA-binding domain of mDia2 compared with the appropriate regions of the p140mDia (17) and Drosophila Diaphanous (41) proteins.

Figure 2A shows a comparison of the ROCK RhoA binding regions with Kinectin cDNA clone D2.

Figure 2B shows a comparison of the ROCK-I/Kinectin homology region with sequences in ROCK-II, $\beta 2$, Skn7 and Kinectin (N-terminal sequence).

35 Detailed Description of the Invention
Definitions

Skn7 Polypeptide

- The term "Skn7 polypeptide" includes a polypeptide including the amino acid sequence for *Saccharomyces cerevisiae* wild-type Skn7 shown in Brown et al., 1993 (J. Bacteriol., 175, 6908-6915) and variants thereof (which may be naturally occurring or synthetic), as discussed below, in particular showing a characteristic of *S.cerevisiae* Skn7 polypeptide, — such as binding to the specific nucleotide sequence motif bound by *S.cerevisiae* Skn7, discussed below, or a variant thereof, transcription factor activity, particularly ability to activate transcription of genes in the activation of a cellular response to stress, such as oxidative stress and/or heat shock.
- Variants include homologues and analogues which are likely to exist in all fungi, as evidenced by the very close structural identity, close sequence identity in the DNA binding domain, and high level of homology of the homologues/analogues in *S. pombe* and *S.cerevisiae*, which yeast are not closely related.
- Accordingly, the present invention may be applied to any fungus, including pathogens such as *Cryptococcus neoformans*, *Candida albicans* and *Aspergillus* and others including those mentioned above. Part of the *Candida albicans* Skn7 is available on the Internet published by the Stanford DNA Sequence and Technology Centre, at <http://candida.stanford.edu>. This confirms that the Skn7 sequence of *Candida* is conserved with that of *S.pombe* and *S.cerivisiae*, in that there is greater than 75% identity in the region between amino acid residues 377 to 456 of the *C.albicans* and *S.cerivisiae* sequences.

The homologue or analogue of Skn7 of any of these organisms may be used in the present invention.

35 β 2 polypeptide.

The term " β 2 polypeptide" includes a polypeptide which is a subunit of a G protein receptor made up of α , β and γ

polypeptides, with G proteins being associated with a variety of receptors such as dopamine, muscarinic, β -adrenergic and adenylyl cyclase. The β 2 protein is described by Gao et al, PNAS 84; 6122 (1987) and its sequence can be found as Genbank
5 accession number M36429.

Rho polypeptide. -

The term "Rho polypeptide" includes a member of the Rho family of GTPases and variants thereof (which may be
10 naturally occurring or synthetic), as discussed below. This includes a protein showing a characteristic of mammalian RhoA, including the ability to interact with the ROCK/ROK protein kinases and/or one or more other mammalian RhoA effectors described herein. The sequence of human RhoA may be
15 found at X05026 / M25080. The protein is also described by Yeriman et al, NAR, 15; 1869 (1987). It further includes a protein showing the characteristic of *S.cerevisiae* Rho1, including the ability to interact with PKC1 (see Nanaka et al, EMBO J., 14; 5931-5938 (1995) and Kamada et al, J. Biol.
20 Cham., 271; 9193-9196 (1996)) and/or control the activity of $\beta(1\rightarrow3)$ glucan synthetase. Variants include homologues and analogues which exist in other eukaryotic cells, particularly mammalian cells or other yeast cells. The sequence of Rho1 may be found at Genbank accession no. M15189, see also
25 Madaule et al, PNAS 84; 779-783, (1987). Rho1 of *C.albicans* is disclosed in Kondoh et al, J. Bacteriol., (1997) 179; 7734-41.

For the purposes of the present invention, the term "Rho
30 polypeptide" also includes the other Rho family members and the Cdc42 protein, as well as their variants.

For example, RHO2 from *S.cerevisiae* is also described by Madaule et al (*ibid*) and RHO3 and RHO4 from *S.cerevisiae* are
35 described by Matsui and Toh-e, (1992), Gene, 114; 43-49. The Genbank accession numbers of these genes are M15190, D10006 and D10007 respectively. *S.cerevisiae* CDC42 is described by

Johnson and Pringle, (1990), J. Cell. Biol. 111; 143-152 and has the Genbank accession number X51906. Human Cdc42 is described by Munemitsu et al, (1990) Mol. Cell. Biol. 10; 5977-5882, and has the Genbank accession number M35543.

- 5 Other Cdc42 genes from other species are known and may also be used.

Variants

- 10 Variants of the above-described polypeptides may be mutants, such as temperature sensitive mutants, alleles such as sequence variants of the proteins described above which demonstrate a substantially similar phenotype, homologues and analogues, such as found in other species, or synthetic variants and derivatives, which retain the functions of the
- 15 above described proteins to the extent necessary for the particular assay format being utilised. Those of skill in the art will appreciate that not all the functional features of the wild-type polypeptides will be required in all the assay formats described herein.

- 20 Thus instead of using the wild-type Skn7, β 2 or Rho polypeptides referenced above in various aspects and embodiments of the present invention, one may include an amino acid sequence which differs by one or more amino acid
- 25 residues from the wild-type amino acid sequence, by one or more (e.g. from 1 to 20, such as 2, 3, 5 or 10) of addition, insertion, deletion and substitution, preferably substitution, of one or more amino acids. Thus, variants, derivatives, alleles, mutants and homologues, e.g. from other
- 30 organisms, are included. Variants of the Rho polypeptides include dominant active mutants such as RhoA(G14V) which keeps the protein in its GTP bound form. Variants also include mutations which destroy the CAAX motif and thereby disrupt membrane targeting. Other types of variant are (i)
- 35 those that block the ability of exchange factors to exchange GTP for GDP by binding to nucleotide exchange factors, preventing activation of the GTPase (e.g. RhoT19N, reviewed

by Quilliam et al, BioEssays 17; 395 (1995)), (ii) those that facilitate ready nucleotide exchange (e.g. F28L; in RhoA F30L (Lin et al, Curr Biol 7; 794 (1997))). Based on the substantial sequence identity between human RhoA and yeast Rho1, these changes may also be introduced into the yeast protein, e.g RhoA G14V is equivalent to Rho1 G19V.

Preferably, a Skn7 amino acid sequence of a variant shares homology with the *S. cerevisiae* Skn7, a β 2 amino acid sequence of a variant shares homology with the Genbank M16538 sequence, and a RhoA and Rho1 variant share homology with Genbank X05026 or M15189 sequences respectively. "Shares homology" refers to a degree of amino acid identity of at least about 30%, or 40%, or 50%, or 60%, or 70%, or 80%, or more preferably at least about 90% or 95% identity.

Variants also include fragments of wild type or variant Skn7 or β 2 polypeptides since our findings make it unnecessary to use the entire protein for assays of the invention. Fragments may be any suitable size, for example from 20 to 300 amino acids, for example from 100 to 200 amino acids. Similarly, fragments of the Rho polypeptide may also be used, again preferably of these size ranges. Generally, it is preferred that substantially intact Rho proteins, which are about 200 amino acids in size, be used.

Fragments may be generated and used in any suitable way known to those of skill in the art. Suitable ways of generating fragments include, but are not limited to, recombinant expression of a fragment from encoding DNA. Such fragments may be generated by taking encoding DNA, identifying suitable restriction enzyme recognition sites either side of the portion to be expressed, and cutting out said portion from the DNA. The portion may then be operably linked to a suitable promoter in a standard commercially available expression system. Another recombinant approach is to

amplify the relevant portion of the DNA with suitable PCR primers. Small fragments (up to about 20 or 30 amino acids) may also be generated using peptide synthesis methods which are well known in the art. Fragments of Skn7 or $\beta 2$ polypeptides may be tested for the ability to bind to a Rho polypeptide, particularly wild type RhoA or Rho1, and these fragments selected for use in the present invention. The fragments will be those which retain the homology domain described herein.

The ability of suitable fragments of Skn7 or $\beta 2$ polypeptide to bind to a Rho polypeptide (or fragment thereof), or suitable fragments of a Rho polypeptide to bind to a Skn7 or $\beta 2$ polypeptide (or fragment thereof), may be tested using routine procedures such as those illustrated in the accompanying examples.

A polypeptide consisting essentially of a ROCK/Kinectin Homology Domain.

This refers to a polypeptide which contains the domain we have identified without the flanking sequences from the ROCK/ROC or Kinectin domains with which it is naturally associated. The polypeptide is desirably from 8 to 40 amino acids in size, and comprises core motif of formula (A):

$LX^6X^7X^8X^9X^{10}X^{11}L$ (A)

wherein:

X^6 is R, A or K;

X^7 is X (where X is any amino acid);

X^8 is E or R;

X^9 is X;

X^{10} is D or E; and

X^{11} is X;

or a variant thereof in which up to 2, preferably 0 or 1, of the residues X^6 , X^8 or X^{10} may be substituted by another amino acid.

Where the polypeptide comprises from 9 to 40 amino acids, the 1 to 32 amino acids additional to this sequence may be at the N-terminal, C-terminal, or any combination thereof. In one preferred aspect, the invention provides polypeptides which
 5 consist essentially of the amino acid sequence $LX^6X^7X^8X^9X^{10}X^{11}L$ as defined above.

In another aspect, the polypeptide may be of from 15 to 40 amino acids and comprise all or part of the sequence of
 10 formula (B):
 $X^1X^2X^3X^4LX^6X^7X^8X^9X^{10}X^{11}LX^{13}X^{14}X^{15}X^{16}X^{17}X^{18}X^{19}X^{20}X^{21}X^{22}$ (B)
 wherein:

- X^1 is D, E, A or T;
- X^2 is ϕ where ϕ is a hydrophobic amino acid;
- 15 X^3 is X;
- X^4 is N, Q, M or A;
- X^6 to X^{11} are as defined above;
- X^{13} is N, Q, E, R or K;
- X^{14} is E, M, N or K
- 20 X^{15} is E, Q, R or K
- X^{16} is ϕ ;
- X^{17} is X;
- X^{18} is X;
- X^{19} is A or S;
- 25 X^{20} is E, Q, R or K;
- X^{21} is X; and
- X^{22} is E or Q;

or a variant thereof in which from 1 to 5 residues other than the leucines at positions 5 and 12 may be substituted by
 30 another amino acid. Preferably from 1 to 4, more preferably from 1 to 3 and most preferably 1 or 2 residues are substituted. The substitutions may be conservative, such that a hydrophobic amino acid is replaced with another hydrophobic amino acid, and a charged amino acid is replaced
 35 by another charged amino acid, preferably of the same charge.

Where the polypeptide comprises part of the sequence (B), it

will comprise the polypeptide of the formula $LX^6X^7X^8X^9X^{10}X^{11}L$, as defined above. Preferably the polypeptide consists of from 22 to 40, such as from 22 to 30, for example from 22 to 25 amino acids. It may also consist essentially of the 22 amino acids set out above or a fragment of from 8 to 22, such as 12, 15 or 18 to 22 amino acids thereof.

Where it comprises from 22 to 40 amino acids, the additional sequences at the N-terminal or C-terminal ends of (B) may include naturally occurring sequences with which the homology domain is normally associated, or synthetic sequences including epitopes, random polypeptide sequences or combinatorially-generated sequences.

By "any amino acid" it is meant one of the 20 naturally occurring amino acids encoded by genetic code. Each separate occurrence of X and ϕ in the peptide may be independently selected.

By "hydrophobic amino acid" it is meant G, A, V, L, I, P, F or M.

In an alternative aspect, the polypeptide of formula (B) is defined as follows:

- X¹ is D, or E;
- X² is ϕ where ϕ is a hydrophobic amino acid;
- X³ is X;
- X⁴ is N or Q;
- X⁶ is R;
- X⁷ is X;
- X⁸ is E;
- X⁹ is X;
- X¹⁰ is D or E;
- X¹¹ is X;
- X¹³ is N, Q or E;
- X¹⁴ is E or N;
- X¹⁵ is E or Q;

- X¹⁶ is ϕ ;
X¹⁷ is X;
X¹⁸ is X;
X¹⁹ is A or S;
5 X²⁰ is E or Q;
X²¹ is X; and
X²² is E or Q.

In a further alternative aspect, the polypeptide of formula
10 (B) is defined as follows:

- X¹ is D, E, A or T;
X² is ϕ where ϕ is a hydrophobic amino acid;
X³ is X;
X⁴ is N or Q;
15 X⁶ is R;
X⁷ is R;
X⁸ is R;
X⁹ is X;
X¹⁰ is D or E;
20 X¹¹ is X;
X¹³ is N or Q;
X¹⁴ is K;
X¹⁵ is E or Q;
X¹⁶ is ϕ ;
25 X¹⁷ is X;
X¹⁸ is X;
X¹⁹ is S;
X²⁰ is K;
X²¹ is X; and
30 X²² is E.

There is also provided a polypeptide of formula (C):

AFGNLRRRVVDKLQKELDMSKME

(C)

- or a variant thereof in which from 1 to 5 residues other than
35 the leucines at positions 5 and 12 may be substituted by
another amino acid residue. Preferably from 1 to 4, more
preferably from 1 to 3 and most preferably 1 or 2 residues

are substituted. The substitutions may be conservative, such that a hydrophobic amino acid is replaced with another hydrophobic amino acid, and a charged amino acid is replaced by another charged amino acid, preferably of the same charge.

5

As with polypeptides of the formula B, the invention also includes polypeptides which consist of from 22 to 40, such as from 22 to 30, for example from 22 to 25 amino acids which contain the polypeptides of formula (C) and its variants. It may also consist essentially of the 22 amino acids set out above or a fragment of from 8 to 22 amino acids, such as from 12, 15 or 18 to 22 amino acids thereof.

15

Unless specified to the contrary, the polypeptide sequences described herein are shown in the conventional 1-letter code and in the N- terminal to C-terminal orientation. The amino acid sequence of polypeptides of the invention may also be modified to include non-naturally-occurring amino acids or to increase the stability of the compound *in vivo*. When the compounds are produced by synthetic means, such amino acids may be introduced during production. The compound may also be modified following either synthetic or recombinant production.

25

Polypeptides of the invention may also be made synthetically using D-amino acids. In such cases, the amino acids will be linked in a reverse sequence in the C to N orientation. This is conventional in the art for producing such peptides.

30

A number of side-chain modifications for amino acids are known in the art and may be made to the side chains of polypeptides of the present invention. Such modifications include for example, modifications of amino groups by reductive alkylation by reaction with an aldehyde followed by reduction with NaBH_4 , amidination with methylacetimidate or acylation with acetic anhydride.

35

The guanidino groups of arginine residues may be modified by the formation of heterocyclic condensation products with reagents such as 2,3-butanedione or glyoxal. Sulphydryl groups may be modified by methods such as carboxymethylation, tryptophan residues may be modified by oxidation or alkylation of the indole ring and the imidazole ring of histidine residues may be modified by alkylation.

The carboxy terminus and any other carboxy side chains may be blocked in the form of an ester group, e.g. a C₁₋₆alkyl ester. Amino terminal modifications may also be made, for example the amino terminus may be in the form of an alkyl or dialkyl amino group.

The above examples of modifications to amino acids are not exhaustive. Those of skill in the art may modify amino acid side chains where desired using chemistry known per se in the art.

Polypeptides of the invention may be formulated in the form of a salt. Salts of polypeptides of the invention which may be conveniently used in therapy include physiologically acceptable base salts, eg derived from an appropriate base, such as alkali metal (e.g. sodium), alkaline earth metal (e.g. magnesium) salts, ammonium and NR₄ (wherein R is C₁₋₄ alkyl) salts. Salts also include physiologically acceptable acid addition salts, including the hydrochloride and acetate salts.

Polypeptides of the invention may be made synthetically or recombinantly, using techniques which are widely available in the art. Synthetic production generally involves step-wise addition of individual amino acid residues to a reaction vessel in which a polypeptide of a desired sequence is being made. Examples of recombinant techniques are illustrated herein.

Polypeptides of the invention may be in a substantially isolated form. It will be understood that the polypeptide may be mixed with carriers or diluents which will not interfere with the intended purpose of the polypeptide and still be regarded as substantially isolated. A polypeptide of the invention may also be in a substantially purified form, in which case it will generally comprise the polypeptide in a preparation in which more than 90%, e.g. 95%, 98% or 99% of the polypeptide in the preparation is a polypeptide of the invention.

A polypeptide of the invention may be labelled with a revealing label. The revealing label may be any suitable label which allows the polypeptide to be detected. Suitable labels include radioisotopes, e.g. ^{125}I , enzymes, antibodies, polynucleotides and linkers such as biotin. Labelled polypeptides of the invention may be used in diagnostic procedures such as immunoassays in order to determine the amount of a polypeptide of the invention in a sample.

A polypeptide or labelled polypeptide of the invention or fragment thereof may also be fixed to a solid phase, for example the surface of an immunoassay well or dipstick.

Such labelled and/or immobilized polypeptides may be packaged into kits in a suitable container along with suitable reagents, controls, instructions and the like.

Additional Sequences.

The homology domain peptides (A) to (C), as well as the other polypeptides used in the present invention, may be linked at either terminus to additional sequences useful for the provision of the assays described herein. For example, the additional sequences may comprise a polyhistidine or epitope (e.g. HA) tag to serve as a tag for pull-down or similar assays. The additional sequences may alternatively be a functional domain, such as a domain for use in a two-hybrid

assay. As another alternative, the domain may be a marker domain, such as a beta-galactosidase, chloramphenicol acetyl transferase, luciferase or green fluorescent protein sequence.

5

The homology domain sequence may additionally be linked, at the C- or N-terminal, to a member of the class of sequences which are membrane translocation sequences capable of directing a polypeptide through the membrane of a eukaryotic cell. Example of such polypeptides include the HSV-1 VP22 protein (Elliot et al, 1997), the HIV Tat protein (for example residues 1-72 or 37-72, Fawell et al, 1994) or a sequence that is derived from the *Drosophila melanogaster* antennapedia protein. The latter is a peptide containing 16 amino acid residues taken from the third helix of the antennapedia homeodomain protein which translocates across biological membranes (Derossi et al, 1994). This translocation peptide has the sequence:
Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys

20

Amino acid sequence homology and identity.

As is well-understood, homology at the amino acid level is generally in terms of amino acid similarity or identity. Similarity allows for "conservative variation", i.e. substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as arginine for lysine, glutamic acid for aspartic acid, or glutamine for asparagine.

30

The percentage identity of DNA and amino acid sequences to a reference sequence can be calculated using commercially available algorithms. The following programs (provided by the National Center for Biotechnology Information) may be used to determine homologies: BLAST, gapped BLAST, BLASTN and PSI-BLAST, which may be used with default parameters.

35

The algorithm GAP (Genetics Computer Group, Madison, WI). GAP uses the Needleman and Wunsch algorithm to align two complete sequences that maximizes the number of matches and minimizes the number of gaps. Generally, the default
5 parameters are used, with a gap creation penalty = 12 and gap extension penalty = 4. Use of either of the terms "homology" and "homologous" herein does not imply any necessary evolutionary relationship between compared sequences, in keeping for example with standard use of terms such as
10 "homologous recombination" which merely requires that two nucleotide sequences are sufficiently similar to recombine under the appropriate conditions.

Another method for determining the best overall match between
15 a nucleic acid sequence or a portion thereof, and a query sequence is the use of the FASTDB computer program based on the algorithm of Brutlag et al (Comp. App. Biosci., 6; 237-245 (1990)). The program provides a global sequence alignment. The result of said global sequence alignment is
20 in percent identity. Suitable parameters used in a FASTDB search of a DNA sequence to calculate percent identity are: Matrix=Unitary, k-tuple=4, Mismatch penalty=1, Joining Penalty=30, Randomization Group Length=0, Cutoff Score=1, Gap Penalty=5, Gap Size Penalty=0.05, and Window Size=500 or
25 query sequence length in nucleotide bases, whichever is shorter. Suitable parameters to calculate percent identity and similarity of an amino acid alignment are: Matrix=PAM 150, k-tuple=2, Mismatch Penalty=1, Joining Penalty=20, Randomization Group Length=0, Cutoff Score=1, Gap Penalty=5,
30 Gap Size Penalty=0.05, and Window Size=500 or query sequence length in nucleotide bases, whichever is shorter.

Similarity may also be defined and determined by the TBLASTN program, of Altschul et al. (1990) *J. Mol. Biol.* 215: 403-10,
35 which is in standard use in the art. This program may also be used to determine DNA homology. Amino acid identity may also be determined by reference to the Smith-Waterman

algorithm, currently used by the United States Patent and Trademark Office.

A preferred method for determining sequence homology of both DNA and polypeptide sequences is by the use of the gapped BLAST algorithm (Altschul et al, (1997) Nucl. Acid Res. 25 3389-3402) with default parameters, accessed via the internet at <http://www.ncbi.nlm.nih.gov>.

Homology, i.e. identity, may be over the full-length of the relevant polypeptide or may more preferably be over a contiguous sequence of about 15, 20, 25, 30, 40, 50 or more amino acids, compared with the relevant wild-type amino acid sequence.

15

Assay formats.

The identification of the interactions described herein gives rise to the various assays of the invention. Those of skill in the art will be able to provide any number of different assay formats which are based upon these interactions, depending upon a number of circumstances, such as cost, convenience and objectives. For example, some assay formats will be more suited to high throughput methods which are generally designed to be used for primary screens for putative inhibitor compounds. Preferred putative inhibitors identified in such a way may then be subject to other assay formats, for example in vivo formats.

Although the relevant polypeptide may be provided in free form it may also be used in the form of a fusion protein linked to a marker or reporter protein. Assays of the invention may be conducted in the following ways, which are provided by way of illustration and are not limiting:

Two hybrid assays.

One assay format which is widely used in the art to study the interaction of two proteins is a two-hybrid assay. This

assay may be adapted for use in the present invention. A two-hybrid assay comprises the expression in a host cell of the two proteins, one being a fusion protein comprising a DNA binding domain (DBD), such as the yeast GAL4 DNA binding domain, and the other being a fusion protein comprising an activation domain, such as that from GAL4 or VP16. In such a case the host cell will carry a reporter gene construct with a promoter comprising a DNA binding element compatible with the DBD. The reporter gene may be a reporter gene such as chloramphenicol acetyl transferase, His3, luciferase, green fluorescent protein (GFP) and β -galactosidase, with luciferase being particularly preferred.

Two-hybrid assays may be in accordance with those disclosed by Fields and Song, 1989, Nature 340; 245-246. In such an assay the DNA binding domain (DBD) and the transcriptional activation domain (TAD) of the yeast GAL4 transcription factor are fused to the first and second molecules respectively whose interaction is to be investigated. A functional GAL4 transcription factor is restored only when two molecules of interest interact. Thus, interaction of the molecules may be measured by the use of a reporter gene operably linked to a GAL4 DNA binding site which is capable of activating transcription of said reporter gene.

Thus two hybrid assays may be performed in the presence of a potential modulator compound and the effect of the modulator will be reflected in the change in transcription level of the reporter gene construct compared to the transcription level in the absence of a modulator.

Host cells in which the two-hybrid assay may be conducted include mammalian, insect, yeast and bacterial cells, with mammalian and yeast cells (such as *S.cerivisiae* and *S.pombe*) being particularly preferred.

In the case of the present invention, the Skn7, β 2 or

homology domain polypeptide may be fused to a heterologous DNA binding domain such as that of the yeast transcription factor GAL 4. The GAL4 transcription factor includes two functional domains. These domains are the DNA binding domain (DBD) and the transcriptional activation domain (TAD). By fusing $\beta 2$, Skn7 or homology domain polypeptide to one of those domains and the respective counterpart, i.e. a Rho polypeptide, to the other domain, a functional GAL 4 transcription factor is restored only when two proteins of interest interact. Thus, interaction of the proteins may be measured by the use of a reporter gene probably linked to a GAL 4 DNA binding site which is capable of activating transcription of said reporter gene.

15 Pull-down and Immunoprecipitation assays.

The interaction between polypeptides may be studied *in vitro* in a "pull-down" format by labelling one with a detectable label and bringing it into contact with the other which has been immobilised on a solid support, or carries a tag allowing it to be immobilised. Suitable detectable labels include ^{35}S -methionine which may be incorporated into recombinantly produced polypeptides. The recombinantly produced polypeptide may also be expressed as a fusion protein containing an epitope (e.g. an HA tag) which can be labelled with an antibody. Such an antibody may be labelled with a detectable lable, such as an enzyme, for example alkaline phosphatase or horse radish peroxidase.

The protein which is, or is to be, immobilized on a solid support may be immobilized using an antibody against that protein bound to a solid support or via other technologies which are known *per se*. A preferred *in vitro* interaction may utilise a fusion protein including glutathione-S-transferase (GST). This may be immobilized on glutathione agarose beads. In an *in vitro* assay format of the type described above the putative modulator compound can be assayed by determining its ability to diminish the amount of labelled polypeptide which

binds to the immobilized GST-polypeptide counterpart. This may be determined by fractionating the glutathione-agarose beads by SDS-polyacrylamide gel electrophoresis.

Alternatively, the beads may be rinsed to remove unbound
5 protein and the amount of protein which has bound can be determined by counting the amount of label present in, for example, a suitable scintillation counter.

Similarly, such an assay according to the present invention
10 may also take the form of an *in vivo* assay wherein the interaction is studied by way of immunoprecipitation of a Skn7, $\beta 2$ or homology domain polypeptide on the one hand, or a Rho polypeptide on the other. The amount of counterpart polypeptide (i.e. Rho polypeptide when Skn7/ $\beta 2$ /homology
15 domain polypeptide is immunoprecipitated, or vice versa) may be examined by any suitable means, for example Western blotting of the immunoprecipitate and probing with the appropriate antibody.

20 The *in vivo* assay may be performed in a cell line such as a yeast strain in which Skn7 polypeptide and/or $\beta 2$ or a Rho polypeptide is expressed from a vector introduced into the cell.

25 Another assay format is dissociation enhanced lanthanide fluorescent immunoassay (DELFI) (Ogata et al, (1992) J. Immunol. Methods 148(1-2) i 15-22). This is a solid phase based system for measuring the interaction of two macromolecules. Typically one molecule (either a Rho
30 polypeptide or a Skn7, $\beta 2$ or homology domain polypeptide) is immobilised to the surface of a multi well plate and the other molecule is added in solution to this. Detection of the bound partner is achieved by using a label consisting of a chelate of a rare earth metal. This label can be directly
35 attached to the interacting molecule or may be introduced to the complex via an antibody to the molecule or to the molecules epitope tag. Alternatively, the molecule may be

attached to biotin and a streptavidin-rare earth chelate used as the label. The rare earth used in the label may be europium, samarium, terbium or dysprosium. After washing to remove unbound label, a detergent containing low pH buffer is added to dissociate the rare earth metal from the chelate. The highly fluorescent metal ions are then quantitated by time resolved fluorimetry. A number of labelled reagents are commercially available for this technique, including streptavidin, antibodies against glutathione-S-transferase and against hexahistidine.

In an alternative embodiment of such assays, one of the polypeptides may be labelled with a fluorescent donor moiety and the other labelled with an acceptor which is capable of absorbing the emission from the donor. This allows an assay according to the invention to be conducted by fluorescence resonance energy transfer (FRET). In this mode, the fluorescence signal of the donor will be altered when the homology domain polypeptide (including Skn7 or $\beta 2$ polypeptides) and a Rho polypeptide interact. The presence of a candidate modulator compound which modulates the interaction will increase or decrease the amount of unaltered fluorescence signal of the donor.

FRET is a technique known per se in the art and thus the precise donor and acceptor molecules and the means by which they are linked to the polypeptides used in the assay may be accomplished by reference to the literature.

Suitable fluorescent donor moieties are those capable of transferring fluorogenic energy to another fluorogenic molecule or part of a compound and include, but are not limited to, coumarins and related dyes such as fluoresceins, rhodols and rhodamines, resorufins, cyanine dyes, bmanes, acridines, isoindoles, dansyl dyes, aminophthalic hydrazines such as luminol and isoluminol derivatives, aminophthalimides, aminonaphthalimides, aminobenzofurans,

aminoquinolines, dicyanohydroquinones, and europium and terbium complexes and related compounds.

5 Suitable acceptors include, but are not limited to, coumarins and related fluorophores, xanthenes such as fluoresceins, rhodols and rhodamines, resorufins, cyanines, difluoroboradiazaindacenes, and phthalocyanines.

10 A preferred donor is fluorescein and preferred acceptors include rhodamine and carbocyanine. The isothiocyanate derivatives of these fluorescein and rhodamine, available from Aldrich Chemical Company Ltd, Gillingham, Dorset, UK, may be used to label the polypeptides used in the assay. For attachment of carbocyanine, see for example Guo et al, J. Biol. Chem., 270; 27562-8, 1995.

Cell lines.

20 The cell lines used in assays of the invention may be used to achieve transient expression, although in a further aspect of the invention cells which are stably transfected with constructs which express Skn7 and/or $\beta 2$ polypeptide and, where required a Rho polypeptide may also be generated. Means to generate stably transformed cell lines are well known in the art and such means may be used here.

25 Systems for cloning and expression of a polypeptide in a variety of different host cells are well known. Suitable host cells include bacteria, mammalian cells and yeast, and baculovirus systems. A common, preferred bacterial host is 30 *E. coli*. Preferred for performance of aspects of the present invention are yeast cells.

Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter 35 sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. Vectors may be plasmids, viral e.g. 'phage, or

phagemid, as appropriate. For further details see, for example, *Molecular Cloning: a Laboratory Manual*: 2nd edition, Sambrook et al., 1989, Cold Spring Harbor Laboratory Press. Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in *Current Protocols in Molecular Biology*, Ausubel et al. eds., John Wiley & Sons, 1992.

Thus a further aspect of the invention provides cells which comprise an expression construct comprising a Rho polypeptide-encoding sequence operably linked to a heterologous promoter, together with an expression construct comprising one of a Skn7 or $\beta 2$ polypeptide-encoding sequence operably linked to a heterologous promoter. The two constructs may be present on separate vectors, or the same vector.

20 Modulator Compounds.

Modulator compounds are those which cause the various interactions described herein which form the basis of the present invention to be altered, e.g. agonised or antagonised. The preferred assays of the invention will be designed for antagonists, i.e. inhibitors, of the interactions.

The amount of putative modulator compound which may be added to an assay of the invention will normally be determined by trial and error depending upon the type of compound used. Typically, from about 10 to 200 μM concentrations of putative modulator compound may be used, for example from 50 to 100 μM .

Modulator compounds which may be used may be natural or synthetic chemical compounds used in drug screening programmes. Extracts of plants which contain several

characterised or uncharacterised components may also be used. Inhibitor compounds may be provided by way of libraries of commercially available compounds. Such libraries, including libraries made by combinatorial chemical means, are available
5 from companies such as Oxford Asymmetry, Oxford, UK; Arqule Inc, MA, USA; Maybridge Limited, Cornwall, UK, and Tripos UK Limited, Bucks, UK. A further class of putative inhibitor compounds can be derived from Skn7 polypeptide, $\beta 2$ polypeptide or Rho polypeptide. Peptide fragments of from 5
10 to 40 amino acids such as those described herein, for example from 6 to 10 amino acids from the region of the relevant polypeptide responsible for interaction between these proteins, or interaction with nucleic acid, may be tested for their ability to disrupt such interaction. Such peptides may
15 further include the homology domain peptides of the present invention.

Antibodies directed to the site of interaction in either protein form a further class of putative inhibitor compounds.
20 Candidate inhibitor antibodies may be characterised and their binding regions determined to provide single chain antibodies and fragments thereof which are responsible for disrupting the interaction.

25 Antibodies may be obtained using techniques which are standard in the art. Methods of producing antibodies include immunising a mammal (e.g. mouse, rat, rabbit, horse, goat, sheep or monkey) with the protein or a fragment thereof. Antibodies may be obtained from immunised animals using any
30 of a variety of techniques known in the art, and screened, preferably using binding of antibody to antigen of interest. For instance, Western blotting techniques or immunoprecipitation may be used (Armitage et al., 1992, Nature 357: 80-82). Isolation of antibodies and/or antibody-producing cells from an animal may be accompanied by a step
35 of sacrificing the animal.

As an alternative or supplement to immunising a mammal with a peptide, an antibody specific for a protein may be obtained from a recombinantly produced library of expressed immunoglobulin variable domains, e.g. using lambda bacteriophage or filamentous bacteriophage which display functional immunoglobulin binding domains on their surfaces; for instance see WO92/01047. The library may be naive, that is constructed from sequences obtained from an organism which has not been immunised with any of the proteins (or fragments), or may be one constructed using sequences obtained from an organism which has been exposed to the antigen of interest.

Antibodies according to the present invention may be modified in a number of ways. Indeed the term "antibody" should be construed as covering any binding substance having a binding domain with the required specificity. Thus the invention covers antibody fragments, derivatives, functional equivalents and homologues of antibodies, including synthetic molecules and molecules whose shape mimics that of an antibody enabling it to bind an antigen or epitope.

Example antibody fragments, capable of binding an antigen or other binding partner are the Fab fragment consisting of the VL, VH, C1 and CH1 domains; the Fd fragment consisting of the VH and CH1 domains; the Fv fragment consisting of the VL and VH domains of a single arm of an antibody; the dAb fragment which consists of a VH domain; isolated CDR regions and F(ab')₂ fragments, a bivalent fragment including two Fab fragments linked by a disulphide bridge at the hinge region. Single chain Fv fragments are also included.

A hybridoma producing a monoclonal antibody according to the present invention may be subject to genetic mutation or other changes. It will further be understood by those skilled in the art that a monoclonal antibody can be subjected to the techniques of recombinant DNA technology to produce other

antibodies or chimeric molecules which retain the specificity of the original antibody. Such techniques may involve introducing DNA encoding the immunoglobulin variable region, or the complementarity determining regions (CDRs), of an antibody to the constant regions, or constant regions plus framework regions, of a different immunoglobulin. See, for instance, EP184187A, GB 2188638A or EP-A-0239400. Cloning and expression of chimeric antibodies are described in EP-A-0120694 and EP-A-0125023.

Hybridomas capable of producing antibody with desired binding characteristics are within the scope of the present invention, as are host cells, eukaryotic or prokaryotic, containing nucleic acid encoding antibodies (including antibody fragments) and capable of their expression. The invention also provides methods of production of the antibodies including growing a cell capable of producing the antibody under conditions in which the antibody is produced, and preferably secreted.

Other candidate inhibitor compounds may be based on modelling the 3-dimensional structure of Skn7, β 2 or Rho polypeptides and using rational drug design to provide potential inhibitor compounds with particular molecular shape, size and charge characteristics.

An inhibitor compound identified using the present invention has therapeutic potential. Budding yeast that are deleted for Skn7 are alive but compromised in their response to stress. Invading pathogenic organisms will respond to attack by the host defences through their own defence systems, the intracellular stress response. Neutralising Skn7 function will render the pathogen more sensitive to bodily defences. As noted, the conservation of Skn7 in fungi provides indication that any substance identified with the requisite inhibitor activity will be of therapeutic value against a wide spectrum of fungal pathogens. Furthermore, since Skn7

does not occur in human cells, drugs active against Skn7 function should not harm human cells.

5 Anti-fungal treatment is useful against Candidiasis, Farmers' Lung, Cryptococcosis and opportunistic fungal infections, e.g. as are prevalent in immuno-compromised individuals, such as transplant patients and AIDS sufferers.

10 Inhibitor compounds may also be used in combination with any other anti-fungal compounds, e.g. azole compounds such as fluconazole. In such a case, the assay of the invention, when conducted *in vivo*, need not measure the degree of inhibition of binding or transcriptional activation caused by the inhibitor compound being tested. Instead the effect on
15 fungal cell growth and/or viability may be measured. It may be that such a modified assay is run in parallel or subsequent to the main assay of the invention in order to confirm that any effect on cell growth and/or viability is as a result of the inhibition of binding or transcriptional
20 activation caused by said inhibitor compound and not merely a general toxic effect.

Skn7 DNA binding site.

We have previously determined that Skn7 binds to a 23
25 nucleotide sequence within the TRX2 promoter, and also to a separate sequence found in the SSA1 promoter (see Morgan et al, EMBO J., 16; 1035-1044 (1997) and PCT/GB98/00643). These and other Skn7 binding sequences may also be used in assays of the present invention.

30

The TRX2 promoter sequence comprises:

5' TTTCCAGCCAGCCGAAAGAGGGA. Within this sequence is the motif CGAAA which has previously been identified as an SCB element (see examples). Mutation of the element to ATAAA lowers Skn7
35 binding 20-fold (see examples).

The 26 bp sequence from the HSE2 region of the SSA1 promoter

has the sequence:

5' TGCATTTTCCAGAACGTTCCATCGGC.

Particular alterations of these sequences abolish Skn7
5 binding. The minimal sequence to which Skn7 binds may be
identified by using synthetic oligonucleotides of these
regions as competitor DNA in a gel mobility shift assay.
Systematic mutation of the nucleotide sequences will define
key residues. In another approach, randomly generated
10 oligonucleotides may be passed over immobilised, pure Skn7
protein. Elution of bound oligonucleotides followed by DNA
sequencing will directly reveal the sequences.

Thus sequences which comprise a Skn7 binding site may be
15 mutants, variants, derivatives or homologues of the above
sequences by way of addition, deletion, substitution and/or
insertion of one or more base pairs, provided such sequences
retain Skn7 binding. The sequences may be shorter sequences
than these 23 and 26 base pair sequences shown. Such
20 variants, mutants, derivatives or homologues may have at
least about 50%, 60%, 70%, 80%, 90% or 95% homology with the
sequences shown.

In particular, the core triplet GAA appears to be a potential
25 core recognition sequence. While not wishing to be bound by
any one particular theory, preferred sequences comprise
derivatives of the above sequences which comprise this core,
are at least 15, preferably 18, nucleotides in length, and
are at least 75% homologous and preferably no more than
30 three, more preferably no more than two nucleotides different
from either of the above sequences when aligned to the GAA
core.

The assays of the invention may utilise a promoter construct,
35 able to activate transcription of an operably linked
sequence, including a Skn7 binding sequence as defined above
including a said mutant, variant, derivative or homologue

thereof. Thus, a Skn7-binding site may be used in construction of a promoter that contains one or more other regulatory motifs, transcription factor elements, and promoter elements to produce a promoter which contains a heterologous Skn7-polypeptide-binding site.

The invention further provides the use of the above constructs and oligonucleotides in an assay to determine the binding of a Skn7 polypeptide to a Rho polypeptide, for example in the presence and absence of a putative inhibitor compound.

By "promoter" is meant a sequence of nucleotides from which transcription may be initiated of DNA operably linked downstream (i.e. in the 3' direction on the sense strand of double-stranded DNA).

"Operably linked" means joined as part of the same nucleic acid molecule, suitably positioned and oriented for transcription to be initiated from the promoter. DNA operably linked to a promoter is "under transcriptional initiation regulation" of the promoter.

Nucleic acid constructs comprising a promoter responsive to Skn7 polypeptide binding, by virtue of including a Skn7-polypeptide-binding motif, and a heterologous gene (reporter) may be employed in screening for a substance able to modulate activity of the promoter by interference with Skn7-polypeptide binding to a Rho polypeptide. "Promoter activity" is used to refer to ability to initiate transcription. The level of promoter activity is quantifiable for instance by assessment of the amount of mRNA produced by transcription from the promoter or by assessment of the amount of protein product produced by translation of mRNA produced by transcription from the promoter. The amount of a specific mRNA present in an expression system may be determined for example using specific oligonucleotides which

are able to hybridise with the mRNA and which are labelled or may be used in a specific amplification reaction such as the polymerase chain reaction. Use of a reporter gene facilitates determination of promoter activity by reference
5 to protein production.

Generally, the gene may be transcribed into mRNA which may be translated into a peptide or polypeptide product which may be detected and preferably quantitated following expression. A
10 gene whose encoded product may be assayed following expression is termed a "reporter gene", i.e. a gene which "reports" on promoter activity.

A reporter gene preferably encodes an enzyme which catalyses
15 a reaction which produces a detectable signal, preferably a visually detectable signal, such as a coloured product. Many examples are known, including β -galactosidase and luciferase. β -galactosidase activity may be assayed by production of blue colour on substrate, the assay being by eye or by use of a
20 spectrophotometer to measure absorbance. Fluorescence, for example that produced as a result of luciferase activity, may be quantitated using a spectrophotometer. Radioactive assays may be used, for instance using chloramphenicol acetyl-transferase, which may also be used in non-radioactive
25 assays. The presence and/or amount of gene product resulting from expression from the reporter gene may be determined using a molecule able to bind the product, such as an antibody or fragment thereof. The binding molecule may be labelled directly or indirectly using any standard technique.

30 Those skilled in the art are well aware of a multitude of possible reporter genes and assay techniques which may be used to determine gene activity. Any suitable reporter/assay may be used and it should be appreciated that no particular
35 choice is essential to or a limitation of the present invention.

For therapeutic purposes, e.g. for treatment of a yeast or other fungal infection a substance able to down-regulate expression of the promoter may be sought. A method of screening for ability of a substance to modulate activity of a promoter may comprise contacting an expression system, such as a host cell, containing a nucleic acid construct as herein disclosed with a test or candidate substance and determining expression of the heterologous gene.

The level of expression in the presence of the test substance may be compared with the level of expression in the absence of the test substance. A difference in expression in the presence of the test substance indicates ability of the substance to modulate gene expression. An increase in expression of the gene compared with expression of another gene not linked to a promoter as disclosed herein indicates specificity of the substance for modulation of the promoter.

A promoter construct may be introduced into a cell line using any technique previously described to produce a stable cell line containing the reporter construct integrated into the genome. The cells may be grown and incubated with test compounds for varying times. The cells may be grown in 96 well plates to facilitate the analysis of large numbers of compounds. The cells may then be washed and the reporter gene expression analysed. For some reporters, such as luciferase the cells will be lysed then analysed.

Rho-interacting Domain Assays.

In a further aspect of the present invention, there is provided means to identify further cellular proteins or other components which interact with the homology domain identified herein.

Thus polypeptides which consist essentially of the homology domain region (such as peptides (B) and (C) as defined above or peptides of from 22 to 40 amino acids comprising said

domains) may be used as "bait" in a two-hybrid assay to screen for proteins which interact with this domain. More generally, this aspect of the invention comprises bringing such polypeptides into contact with a potential interacting protein and determining whether said protein interacts with the polypeptide. Where the protein interacts, it may be recovered and isolated, and its sequence determined. For example when the protein is isolated by way of a two-hybrid screen, it may be recovered and analysed by means analogous to those of the accompanying examples.

The proteins screened for the ability to interact with the homology domain may be, for example, yeast, insect or vertebrate such as mammalian. Yeast proteins are preferred. Generally the protein will be brought into contact with the homology domain under conditions suitable for RhoA/Rho1 and the domain to interact.

In a related aspect, the homology domain sequences described herein may be used in database homology searches to identify other proteins in yeast or other cells, such as mammalian cells, which interact with Rho polypeptides. Such proteins may then be prepared and tested using methods analogous to those described herein for an interaction with Rho polypeptides and such interactions will form further targets for regulators of the cell cycle. Thus the invention provides a method which comprises searching a sequence database with any of the polypeptide sequences (A), (B) or (C) described herein (including their variants), identifying proteins which comprise a sequence having at least 60%, preferably at least 70%, 80%, 90% or 95% sequence similarity, preparing a protein having said sequence similarity, and testing said protein for its ability to interact with a Rho polypeptide. The invention further provides assays to detect modulators of said interaction which comprise bringing the protein into contact with a Rho polypeptide under conditions in which, in the absence of modulator, the protein and Rho

polypeptide are capable of interacting, and measuring the degree of modulation of the interaction caused by said modulator. Such assays may be conducted by the means described herein.

5

Products and Compositions.

The present invention allows identification of novel substances which can target and disrupt or otherwise modulate the interaction between a Rho polypeptide and the other components described herein. A substance identified by means of the invention may be investigated further. Furthermore, it may be manufactured and/or used in preparation, i.e. manufacture or formulation, of a composition such as a medicament, pharmaceutical composition or drug. These may be administered to individuals.

Thus, the present invention extends in various aspects not only to such substances in accordance with what is disclosed herein, but also a pharmaceutical composition, medicament, drug or other composition comprising such a substance, a method comprising administration of such a composition to a patient, e.g. for anti-fungal treatment, which may include preventative treatment, use of such a substance in manufacture of a composition for administration, e.g. for anti-fungal treatment, and a method of making a pharmaceutical composition comprising admixing such a substance with a pharmaceutically acceptable excipient, vehicle or carrier, and optionally other ingredients.

Also encompassed within the scope of the present invention are functional mimetics of peptides (A), (B) and (C) and their fragments and polypeptide extensions which interfere with interactions between Rho polypeptides and the other polypeptides described herein, particularly a Skn7 polypeptide. The term "functional mimetic" means a substance which may not contain an active portion of the relevant amino acid sequence, and probably is not a peptide at all, but

which retains the relevant interfering activity. The design and screening of candidate mimetics is described in detail below.

5 A substance identified using the present invention may be peptide or non-peptide in nature. Non-peptide "small molecules" are often preferred for many *in vivo* pharmaceutical uses. Accordingly, a mimetic or mimic of the substance (particularly if a peptide) may be designed for
10 pharmaceutical use.

The designing of mimetics to a known pharmaceutically active compound is a known approach to the development of pharmaceuticals based on a "lead" compound. This might be
15 desirable where the active compound is difficult or expensive to synthesise or where it is unsuitable for a particular method of administration, e.g. peptides are not well suited as active agents for oral compositions as they tend to be quickly degraded by proteases in the alimentary canal.
20 Mimetic design, synthesis and testing may be used to avoid randomly screening large numbers of molecules for a target property.

There are several steps commonly taken in the design of a
25 mimetic from a compound having a given target property. Firstly, the particular parts of the compound that are critical and/or important in determining the target property are determined. In the case of a peptide, this can be done by systematically varying the amino acid residues in the
30 peptide, e.g. by substituting each residue in turn. These parts or residues constituting the active region of the compound are known as its "pharmacophore".

Once the pharmacophore has been found, its structure is
35 modelled according to its physical properties, e.g. stereochemistry, bonding, size and/or charge, using data from a range of sources, e.g. spectroscopic techniques, X-ray

diffraction data and NMR. Computational analysis, similarity mapping (which models the charge and/or volume of a pharmacophore, rather than the bonding between atoms) and other techniques can be used in this modelling process.

5

In a variant of this approach, the three-dimensional structure of the ligand and its binding partner are modelled. This can be especially useful where the ligand and/or binding partner change conformation on binding, allowing the model to take account of this in the design of the mimetic.

10

A template molecule is then selected onto which chemical groups which mimic the pharmacophore can be grafted. The template molecule and the chemical groups grafted on to it can conveniently be selected so that the mimetic is easy to synthesise, is likely to be pharmacologically acceptable, and does not degrade *in vivo*, while retaining the biological activity of the lead compound. The mimetic or mimetics found by this approach can then be screened to see whether they have the target property, or to what extent they exhibit it. Further optimisation or modification can then be carried out to arrive at one or more final mimetics for *in vivo* or clinical testing.

20

Mimetics of substances identified as having ability to interfere with the interactions described herein are included within the scope of the present invention.

25

Whether it is a polypeptide, antibody, peptide, nucleic acid molecule, small molecule, mimetic or other pharmaceutically useful compound according to the present invention that is to be given to an individual, administration is preferably in a "prophylactically effective amount" or a "therapeutically effective amount" (as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend on

30

35

the nature and severity of what is being treated. Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors.

5

A composition may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated.

10 Pharmaceutical compositions according to the present invention, and for use in accordance with the present invention, may include, in addition to active ingredient, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in
15 the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material will depend on the route of administration, which may be oral, or by injection, e.g. cutaneous, subcutaneous or intravenous.

20

Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may include a solid carrier such as gelatin or an adjuvant. Liquid
25 pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

30

For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH,
35 isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection,

Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

- 5 A polypeptide, peptide or other substance able to interfere with the interactions described herein according to the present invention may be provided in a kit, e.g. sealed in a suitable container which protects its contents from the external environment. Such a kit may include instructions
10 for use.

Further aspects and embodiments will be apparent to those of ordinary skill in the art upon consideration of the above disclosure and the following examples.

- 15 All documents mentioned in this specification are hereby incorporated herein by reference. In this specification, the terms "comprising" and "comprises" are used synonymously with "including" and "includes".

20

Examples.

- To identify potential RhoA effector proteins we conducted a two-hybrid screen for cDNAs encoding proteins that interact with a Gal4-RhoA.V14 fusion protein. In addition to the RhoA
25 effector ROCK-I we identified cDNAs encoding Kinectin, mDia2 (a p140 mDia-related protein), and the guanine nucleotide exchange factor NET. ROCK-I, Kinectin and mDia2 can bind the wildtype forms of both RhoA and Cdc42 in a GTP-dependent manner *in vitro*. Comparison of the ROCK-I and Kinectin
30 sequences revealed a short region of sequence homology that is both required for interaction in the two-hybrid assay and sufficient for weak interaction *in vitro*. Sequences related to the ROCK-I/Kinectin sequence homology are present in heterotrimeric G protein β subunits and in the *S. Cerevisiae*
35 Skn7 protein. We show that β 2 and Skn7 can interact with mammalian RhoA and Cdc42, and yeast Rho1, both *in vivo* and *in vitro*. Functional assays in yeast suggest that the Skn7

ROCK-I/Kinectin homology region is required for its function in vivo.

Members of the Rho family of GTPases regulate diverse cellular processes ranging from cytoskeletal organisation to gene expression and cell transformation. Upon binding GTP, these Ras-like proteins interact with effector proteins to induce downstream signals (for reviews see 1-3). Recent biochemical and genetic studies have identified many potential Rho effectors in both mammalian cells and the budding yeast *Saccharomyces cerevisiae*. Mammalian RhoA interacts with members of the PKN/PRK and ROCK/ROK protein kinase families (4-10). The ROCKs are clearly involved in cytoskeletal rearrangements (6,11-13), but the functions of the PKN/PRK kinases remain obscure. RhoA also interacts with several apparently non-catalytic effectors including Rhophilin, Rhotekin, Citron, the myosin binding subunit of myosin light chain phosphatase (MBS), p140 mDia, Kinectin and p116RIP (9-11,14-18). RhoA interactions with MBS, p140mDia and Kinectin are likely to be involved in contractile events, actin polymerisation and cytokinesis, and motility respectively (11,17,19,20). In *Sacchchromyces cerevisiae*, *RHO1*, an essential gene (21,22), controls activity of $\beta(1\rightarrow3)$ glucan synthase, the enzyme that synthesises cell wall glucan polymers (23,24), and *Bni1*, a gene involved in cytoskeletal events and cytokinesis (25,26). *Rho1* regulates the *PKC1-MPK1* MAP kinase pathway that controls cell wall integrity (27,28) and the *Rlm1* transcription factor (29,30). Interestingly, *Skn7*, a yeast two-component protein, shows genetic interactions with the *PKC1* pathway, and exhibits several properties that suggest that it too may be a *Rho1* effector (31-33).

Sequence elements involved in the interaction between Rho family GTPases and their effectors are of considerable interest since their definition should permit the identification of further potential effector proteins. The

first such motif to be identified was the CRIB (Cdc42/Rac interactive binding) motif, which specifies interaction with Rac1 and Cdc42, but not RhoA, and has the consensus sequence I S x P x(2 or 3) F x H x (2) [HT] [VA] [GDQ] (34). A
5 second motif, REM-1 (Rho effector motif class 1), is found in the N-terminal Rho-binding domains of the RhoA effectors PKN/PRK1, PRK2, rhotekin and rhophilin, and has the consensus [LIF]-x(2)-[EK]-x(2)-[VIL]-x(2)-G-x-[EKR]-[NRQ] (9,14). REM-1 appears specific for RhoA, although PRK2 has also been
10 reported to bind Rac1 (8). Although the REM-1 motif is reiterated three times at the N-terminus of the PKN/PRK kinases, only a single copy is found in rhophilin and rhotekin. REM-1 motifs appear to differ in their ability to discriminate between the GTP- and GDP-bound forms of RhoA.
15 No sequence motifs common to other RhoA effectors have been identified, although the defined Rho-binding regions of citron, Kinectin and the ROCKs are all found in regions of extended α -helical coiled-coil structure.

20 To identify potential RhoA effectors, we conducted a two-hybrid screen with RhoA.V14 as a bait. In addition to ROCK-I, we identified cDNAs encoding Kinectin, a p140 mDia-related protein, and the mouse homolog of the NET1 GEF. Comparison of the ROCK-I and Kinectin sequences revealed a
25 short region of sequence homology that is both required for and sufficient for interaction with RhoA and Cdc42. A similar sequence is found in both heterotrimeric G protein β subunits and the yeast Skn7 protein, and is also required for the interaction of these proteins with RhoA. In addition to
30 defining a RhoA interaction domain, the ROCK-I/Kinectin homology region is required for the function of Skn7 *in vivo*, consistent with the notion that Skn7 represents a novel Rho effector.

35 Experimental Procedures

Plasmids

Plasmids were constructed by standard techniques; details

are available on request. The SKN7 (Genbank Accession number U00485; Ref. 31) and human $\beta 2$ subunit (Genbank Accession number M16538; Ref. 35); coding sequences were obtained by PCR and their DNA sequences confirmed.

5

GAL4 DNA binding domain fusions

Activated 9E10-tagged RhoA and Cdc42 were subcloned from mammalian expression plasmids (36) as NcoI+Xho fragments into pGBT9; the RhoA CAAX motif was inactivated by a C190S mutation introduced by PCR, and that of Cdc42 by truncation generating Cdc42(1-178)ID. Yeast Rho1 was isolated using PCR and Rho1.G19V/C206S constructed by standard techniques.

10

GAL4 Activation domain fusions:

cDNA clones isolated in the two-hybrid screen are summarised in Table 1. For further two-hybrid analyses DNA fragments were inserted into derivatives of pGAD424 and pGAD10 (Clontech). A cDNA encoding a CRIB-domain protein related to MSE55 (34) was used as a specificity control in the two-hybrid assay. The plasmids encode the following sequences C-terminal to the Gal4 activation domain:

15

cDNA clone D1 encodes IWNSDPREFT-(ROCK-I codons 300-1030)-KKKVNSRDL.

cDNA clone D4 encodes IWNSDPRNLPSSP-(ROCK-I codons 456-1028)-VNLERSMNRRY

25

cDNA clone D9 encodes IWNSDPREFT-(ROCK-I codons 349-1025)-GELERSMNRRY.

GAD-ROCK(831-1010) encodes IEFPM-(ROCK-I codons 831-1010)-DLQRFMNRRY.

GAD-ROCK(831-1010)TT is as GAD-ROCK(831-1010) with K1005T/L1006T.

30

GAD-ROCK(831-1010) Δ HR encodes ISRGS-(ROCK-I codons 831-1010)-FQIYES with codons 950-966 deleted.

GAD-ROCK.HR encodes ISRGS-(ROCK-I codons 950-972)-FQIYES.

GAD-Kinectin(1053-1327) (cDNA clones D2, D3) encodes

35

IWNSDPRDLP-(Kinectin codons 1053-1327).

GAD-Kinectin(1053-1327) Δ HR encodes IEFPMGRDLP-(Kinectin codons 1053-1327) with codons 1191-1215 replaced by GS.

- GAD-mDia2(47-257) (cDNA clone D7) encodes mDia2
 IWNSDPREF-(mDia2 codons 47-800)-VNSREIYES.
 GAD-NET (clone D5) encodes IWNSDPRDLP-(15 codons from mNET1
 5'UT)-(mNET1 1-595).
- 5 GAD-SKN7 encodes ISGRS-(SKN7 codons 1-623)
 GAD-SKN7 Δ HR encodes IEF (SKN7 codons 1-623) with codons
 237-260 replaced by G.
 GAD- β 2.WT encodes ISGRS-(Human β 2 codons 1-340).
 GAD- β 2DHR encodes IEFPM-(β 2 codons 24-340).
- 10 GAD- β 2HR encodes ISGRS-(β 2 codons 1-32)-LEIPDL.
 GAD-PKN.N encodes IEFPM (PKN codons 1-511).
 GAD-PAK.N encodes IEFPMAGS-(Rat PAK α codons 1-252)-RRPAEIYES.

GST fusion proteins:

- 15 All fusion proteins were made using pGEX-KG, and encode
 the following sequences:
 pGEX-RhoA.WT and pGEX-Cdc42WT encode wildtype RhoA and Cdc42
 respectively.
 GST-ROCK(831-1010) encodes (ROCK-I codons 831-1010)-LELKLNSS.
- 20 GST-ROCK(831-1010)TT is as GST-ROCK(831-1010) with
 K1005T/L1006T.
 GST-ROCK(831-1010) Δ HR encodes ROCK(831-1010)-ES, with codons
 950-966 deleted.
 GST-ROCK.HR encodes ROCK(950-972)-F.
- 25 GST-Kinectin(1053-1327) encodes Kinectin codons 1053-1372.
 GST-Kinectin(1053-1327) Δ HR is GST-Kinectin(1053-1327) with
 codons 1191-1215 replaced by GS.
 GST-mDia2(47-257) encodes (mDia2 codons 47-257)QLNSS.
 GST-NET encodes mNET1 (codons 122-595).
- 30 GST-SKN7 encodes SKN7 1-623.
 GST-SKN7 Δ HR encodes (SKN7 codons 1-623) with codons 237-260
 replaced by G.
 GST- β 2.WT encodes (β 2 codons 1-340).
 GST- β 2DHR encodes (β 2 codons 24-340).
- 35 GST- β 2HR encodes (β 2 codons 1-32)-LELKLNSS.
 GST-PAK.N encodes (Rat PAK α codons 1-252)-EIRRLELKLNSS.
 GST-PKN.N encodes (PKN codons 1-511).

Two-hybrid screen and yeast manipulations.

Yeast strains and manipulations were as described previously (33,37,38). For the two-hybrid screen, yeast strain HF7c carrying pGBT9-RhoA.V14/S190 was used in conjunction with a mouse T-helper cell cDNA library in pSE1107 (39). 4 million transformants were plated on to selective plates lacking histidine and grown for three days at 30°C. Colonies were rescreened for expression of the LacZ marker after lifting onto nitrocellulose filters (38).

Recombinant proteins

Overnight cultures were diluted 1:10 to 50ml, grown for 3hr, then lysed by sonication in 5ml RB (100mM NaCl, 5mM MgCl₂, 25mM Tris pH 7.2) with protease inhibitors, adsorbed to 0.5ml Glutathione-Sepharose 4B beads and washed extensively. RhoA.WT-9E10 and Cdc42.WT-9E10 proteins were released by overnight incubation with thrombin (5U; Sigma) at 4°C in RB; thrombin was removed by adsorption to p-aminobenzamidine-Sepharose 6B (0.5 ml; Sigma). Protein concentrations were determined by dye-binding assay (Biorad) or by comparison to known standards on Coomassie-stained SDS-PAGE gels.

In vitro binding assay

Equimolar amounts of each GST-fusion protein (~100-300ng) were bound to glutathione-sepharose beads and incubated with 10 ng of GTP-γ-S- or GDP-loaded RhoA.WT-9E10 or Cdc42.WT-9E10 at 4°C for two hours, with agitation, in RB containing 0.5 mg/ml bovine serum albumin. The beads were then washed in ice-cold RB / 0.1% NP-40, and bound GTPase eluted by boiling in SDS-PAGE sample buffer. Following fractionation by SDS-PAGE, GTPases were detected by immunoblotting with the 9E10 antibody.

Results

Identification of potential RhoA effectors by the two-hybrid screen.

We performed a two-hybrid screen for proteins that can interact with the activated form of RhoA. A fusion gene, Gal4-RhoA.V14/S190, was constructed in which the Gal4 DNA binding domain is fused N-terminal to activated human RhoA (RhoA.V14), carrying an additional mutation at its C-terminus to destroy the CAAX motif. Yeast HF7c cells expressing Gal4-RhoA.V14/S190 were used to screen a library of Gal4 activation domain-tagged mouse T-cell cDNA. Seven transformants exhibited Gal4-RhoA.V14/S190-dependent activation of both the His3 and LacZ markers in HF7c cells. The cDNAs were characterized by partial DNA sequencing. Table 1 lists these cDNAs and provides Genbank Accession numbers(given in brackets). The residues encoded by each insert are shown and it was noted that the same Kinectin cDNA was identified in two independent transformants. The N-terminal residue of the mDia2 sequence is assigned by comparison with a mouse EST (Genbank accession number AA415402; Figure 1), and its C-terminal residue by comparison with the p140mDia sequence.

Plasmids D1, D4 and D9 carry partial cDNAs encoding the RhoA effector kinase ROCK-I (p160ROCK; Ref.7). Plasmids D2 and D3 carried the same partial Kinectin cDNA (20); interestingly, although Kinectin was previously identified as a putative RhoA effector, the cDNA fragment isolated in our screen does not overlap that isolated in the previous screen (15; see Discussion). Plasmid D5 carried a complete open reading frame 83% identical to the putative Rho-family guanine-nucleotide exchange factor NET1 (40). Plasmid D7 contains a cDNA related to p140mDia, identified in a previous screen for RhoA effector proteins, (17) which is related to the Drosophila gene *Diaphanous* (41). In vitro experiments described below indicated that the RhoA-binding domain of this protein is located in its N-terminal sequences; we therefore sequenced this region, which spans codons 47-257, and compared it with p140mDia and with Drosophila *Diaphanous*.

Figure 1 shows the sequence compared with the appropriate regions of the p140mDia (17) and *Drosophila Diaphanous* (41) proteins. The asterisk in figure 1 marks the start of the mDia2 sequences isolated in clone D7. Identical residues in two or more of the sequences are reverse-shaded, while similar residues are highlighted.

RhoA and Cdc42 bind to Kinectin, ROCK-I, mDia-2 in a GTP-dependent manner.

We used the two-hybrid assay to investigate the interactions between RhoA, its yeast homolog Rho1, and human Cdc42 and the proteins identified in the screen, in each case using Gal4-GTPase fusion proteins containing activating mutations and mutated CAAX motifs.

Interaction with RhoA.V14, Cdc42.V12 and Rho1.V19 was tested with fusion constructs from each cDNA (Kinectin (1053-1327), mNET (1-595), ROCK-I (831-1010) and mDia-2 (47-257). HF7C yeast were transformed with combinations of expression plasmids expressing GAL4-DNA binding domain / GTPase or Gal4 activation domain / effector protein fusion genes. Three independent transformants of each combination were assayed for growth after three days at 30°C on selective medium lacking histidine and containing 2mM aminotriazole.

Growth was observed for each transformant. This showed that each of the three GTPases (RhoA, Cdc42 and Rho1) could interact with all the proteins in the assay.

To confirm the specificity of the interactions with Cdc42, we examined the interaction of an MSE55-related protein isolated in a screen for Cdc42 effectors (34); this CRIB motif-containing protein interacted with Cdc42.V12, but not RhoA.V14, in the two-hybrid assay, in agreement with previous results.

The two-hybrid data suggest that each of the cDNAs isolated in the screen encodes a protein that can interact with activated forms of both RhoA and Cdc42. To confirm that the wildtype GTPases can also interact with these proteins, and to investigate whether binding is GTP-dependent, we

performed *in vitro* binding assays. GST fusion genes carrying each potential Rho effector were constructed as follows: Kinectin (1053-1327), ROCK-I (831-1010), mDia2 (47-257), and mNET (1-595). To assess the specificity of the assay, we
5 also tested the fusion proteins PKN(1-511), and PAK(1-252) which specifically bind RhoA and Cdc42 respectively (9,42). Recombinant 9E10 epitope-tagged RhoA and Cdc42 proteins were purified from bacteria and loaded with either GTP- γ -S or GDP. Each GTPase (10ng) was incubated with glutathione beads
10 carrying equimolar amounts of one of the GST fusion proteins (100ng to 300ng). Following washing, bound proteins were eluted and fractionated by SDS-PAGE. GTPases were detected by immunoblot with the 9E10 antibody.

Binding of PKN(1-511) and PAK(1-252) was observed to be
15 specific for the GTP-bound forms of RhoA and Cdc42 respectively, demonstrating that our assay conditions allow discrimination between the two GTPases. The RhoA-binding regions from Kinectin, ROCK-I and mDia2 were observed to bind both wildtype RhoA and wildtype Cdc42 in a GTP-dependent
20 manner. In contrast, although binding of mNET to Cdc42 was GTP-dependent, it bound RhoA.GTP and RhoA.GDP equally well. Taken together with the two-hybrid data, these results show that Kinectin, ROCK-I and mDia2 all represent potential effectors for both RhoA and Cdc42; we have also found that
25 mNET is a RhoA GEF.

mROCK-I and Kinectin have similarities within their Rho-binding domains.

The RhoA effectors rhotekin, rhophilin and PKN share a
30 region of homology within their Rho-binding domains (The REM-1 motif; 14). Previous studies of the ROCK proteins have defined a short region within its C-terminal coiled-coiled region that suffices for interaction with RhoA (4,5,43). We therefore compared the sequence spanning this region with the
35 sequences of Kinectin, mDia2, and mNET to identify potential Rho-binding sequence motifs (Fig 2A). Although the maximum homology between the ROCK proteins corresponds to ROCK-I

residues 995-1014 (5,7) a region of substantial similarity between ROCK-I and Kinectin is found N-terminal to this, corresponding to ROCK-I residues 950-972. This region lies within the "leucine zipper" region of ROCK-I and is also within a coiled-coil region in Kinectin. No substantial sequence homology was found with mDia2. We used the homology between ROCK-I and Kinectin to screen the sequence databases for similar sequences using the Blastp program (NCBI). Among many coiled-coil proteins identified, this search detected a second Kinectin sequence element N-terminal that in our cDNA clone (residues 832-854), and a region at the N-terminus of heterotrimeric G protein β subunits. We also observed that the sequence of the *S.cerevisiae* *SKN7* gene, which interacts genetically with the *RHO1-PKC1* pathway (31-33), also contains a region of similarity to the ROCK-I/Kinectin homology, again within a region predicted to form a coiled-coil structure. These sequences are compared in Figure 2B. Similarity between four or more of the sequences is shown in figure 2B by shading, identity by reverse shading.

The ROCK-I/Kinectin homology is required for interaction with Rho proteins

To investigate the significance of the ROCK-I/Kinectin homology, we examined its role in the interactions with RhoA and Cdc42 using both two-hybrid assays and *in vitro* biochemical assays. To facilitate mutagenesis of ROCK-I we examined a shorter ROCK-I fragment containing codons 831-1010, which interacts strongly with RhoA.V14 and Cdc42.V12 in the two-hybrid assay (Table 2, row 2) and with GTP-loaded RhoA and Cdc42 in the *in vitro* binding assay. Sequences encompassing the homology were deleted from both ROCK-I and Kinectin and the interactions of the resulting proteins with RhoA and Cdc42 examined. Proteins lacking the ROCK-I/Kinectin homology region are denoted DHR. Interaction strength was assessed by growth on histidine-selective medium containing increasing amounts of 3-aminotriazole. Scores 1,2,3,4 and 5 correspond to growth on plates containing 0, 1,

2, 4 or 8 mM aminotriazole, respectively. The results are shown in Table 2.

In the plate growth assay, HF7C yeast were transformed with combinations of plasmids expressing GAL4-DNA binding domain / GTPase fusions(RhoA.V14, Rholp.V19) and plasmids expressing Gal4 activation domain / effector protein fusions(Kin(1053-1327), Kin(1053-1327)DHR, β -2, β -2DHR, SKN7, SKN7DHR). Two independent transformants in each case were assayed for growth after three days at 30°C on selective medium lacking histidine and containing 2mM 3-aminotriazole.

Growth was only observed for transformants expressing either RhoA.V14 or Rholp.V19 along with an effector protein fusion containing an intact ROCK-I/Kinectin homology region.

In vitro interactions with RhoA.V14 and Cdc42.V12 were investigated by preloading 9E10-tagged wildtype RhoA or Cdc42 with either GTP-g-S or GDP. Each GTPase (10ng) was incubated with glutathione-beads carrying equimolar amounts of the indicated GST-effector fusion proteins. Following washing, bound GTPases were eluted and fractionated by SDS-PAGE. GTPases were detected by immunoblotting using the 9E10 antibody. The total GTPase input protein was also immunoblotted.

Both RhoA and Cdc42 were detected by immunoblotting in those assays in which the GST fusion derivative contained an intact ROCK-I/Kinectin homology region. This indicates that both RhoA and Cdc42 interact with effector protein when this region is present.

Deletion of the homology region (codons 950-972) from ROCK-I (831-1010) generates a protein which does not interact with RhoA or Cdc42 in either assay (ROCK-I (831-1010) Δ HR: Table 2, row 3). Similarly deletion of the homology region from Kinectin also abolished interaction with RhoA and Cdc42 in both assays (Kinectin(1053-1327) Δ HR: Table 2, row 7). The ROCK-I/Kinectin homology region is therefore required for interaction of both proteins with RhoA and Cdc42.

We also examined whether Skn7 and heterotrimeric G

protein β subunit, which contain sequences related to the ROCK-I/Kinectin homology, also interact with Rho-family GTPases. Sequences encoding $\beta 2$ and Skn7 were amplified by PCR and inserted into appropriate plasmids for use in the two-hybrid and *in vitro* interaction assays. In addition, derivatives of each protein were constructed in which the ROCK-I/Kinectin homology region was deleted (Skn7DHR and $\beta 2$ DHR). In the two-hybrid assay, both proteins interacted with RhoA.V14, yeast Rho1.V19, and Cdc42.V12; deletion of the homology region from either protein reduced interactions with all GTPases to background levels (Table 2, rows 8-11). Both $\beta 2$ and Skn7 also bound wildtype RhoA.GTP and Cdc42.GTP in the *in vitro* binding assay, and all interactions were abolished by deletion of the ROCK-I/Kinectin homology region. Neither protein interacted with GDP-bound GTPases. Thus, both $\beta 2$ and Skn7 interact with GTP-bound Rho proteins.

The homology region is sufficient for interaction with RhoA or Cdc42

We next tested whether the ROCK-I/Kinectin homology region is sufficient for binding to RhoA and Cdc42. Sequences encompassing the homology from ROCK (codons 950-972) or $\beta 2$ (codons 1-32) were inserted into appropriate plasmids for use in the two-hybrid and *in vitro* interaction assays. In addition to deletion of the ROCK-I/Kinectin homology region, previous studies have demonstrated that point mutations of ROK- α (ROCK-II) residues C-terminal to the homology, severely impair its interaction with RhoA in GTPase overlay assays (5). We therefore constructed an analogous mutant, ROCK-I(831-1010)TT, and compared its binding properties with those of the isolated ROCK-I/Kinectin homology region.

In the two-hybrid assay ROCK-I(950-972), the isolated ROCK homology region peptide, interacted with both RhoA.V14 and Cdc42.V12, as did ROCK-I(831-1010)TT (Table 2, rows 4,5). The N-terminal region of $\beta 2$ was also sufficient for interaction with both GTPases in this assay (Table 2, row

12). Similar results were obtained in the *in vitro* binding assay. Immunoblotting indicated a strong interaction between both GTPases and GST fusion derivatives carrying ROCK-1(831-1010) and β -2. GST fusion derivatives carrying ROCK-I(950-972), the isolated ROCK-I/Kinectin homology region peptide, and ROCK-I(831-1010)TT interacted weakly with both GTPases; while deletion of the ROCK-I/Kinectin homology reduced interaction to background levels. A GST derivative carrying β 2(1-32) was sufficient for interaction with both GTPases *in vitro*. Control experiments with PKN(1-511) and PAK(1-252) confirmed that the assay conditions allow discrimination between RhoA- and Cdc42/Rac-specific effector proteins. Taken together, these data suggest that the ROCK-I/Kinectin homology is an essential structure mediating interactions with these GTPases (see Discussion).

The ROCK-Kinectin homology region is required for Skn7 function

The results presented in the preceding section provide strong evidence that Skn7 interacts with both RhoA and Rho1, and show that the ROCK-I/Kinectin homology region is required for this interaction. We therefore examined the role of the ROCK-I/Kinectin homology in Skn7 function in yeast using a number of different assays.

These assays rely either on measuring the effects of Skn7 overexpression in different genetic backgrounds, or on measuring the ability of different Skn7 mutants to suppress the effects of *SKN7* deletions. High level overexpression of *SKN7* from the *GAL1* promoter in wildtype cells is lethal, probably owing to weakening of the cell wall (33). Skn7 overexpression from high copy-number plasmids also activates the MCB promoter element which is partly responsible for G1 cyclin gene expression: Skn7 overexpression can therefore bypass the normal requirement for the *SWI4* and *SWI6* gene products, allowing growth of *swi4^{ts} swi6D* cells at the nonpermissive temperature (33). In addition, Skn7 overexpression partially suppresses the temperature-sensitive

phenotype of cells expressing human RhoA, allowing them to grow at 35.5°C; in contrast, it exacerbates the severity of the temperature sensitive *cdc42* mutation, preventing growth at 35.5°C. Deletion of *SKN7* has a number of effects,

- 5 rendering cells acutely sensitive to oxidative stress (44) and preventing growth of *pkc1-8* cells at 37°C (32,33,37). Moreover, deletion of *SKN7* prevents suppression of the *swi4^{ts} swi6D* double mutation by *Mbp1* overexpression.

- For these studies we constructed a yeast expression
10 plasmid carrying a derivative of the full length *SKN7* ORF, *Skn7ΔHR*, which lacks residues 237-259, spanning the ROCK-I/Kinectin homology region. As controls, we examined wildtype *Skn7* and two other mutants that affect *Skn7*
15 function. One, *Skn7D427N*, lacks the phosphoacceptor aspartate in the receiver domain which is essential for *Skn7* function in the cell cycle (33) but not in the response to free radical stress (44). The other, *Skn7Δ(353-623)*, contains a C-terminal deletion that inactivates the protein. Each of the mutant proteins could be detected by
20 immunoblotting of yeast cell extracts with *Skn7* antiserum, so defects in their function cannot be attributed to changes in protein stability (data not shown).

Table 3 summarises the results of these assays.

- Column A shows high overexpression of *SKN7* from the GAL
25 promoter (33).

Column B shows a reporter gene assay with a *lacZ* reporter controlled by three MCB elements. High copy *SKN7* activates this reporter in a *swi6D* genetic background (33). + indicates blue colour.

- 30 Column C shows suppression of the *swi4^{ts} swi6D* strain K2003 which is temperature-sensitive for growth at 37°C. High copy *SKN7* suppresses this by stimulating G1 cyclin gene expression (33). + indicates growth at 37°C.

- Column D shows expression in strain YOC725, which
35 expresses human RhoA. High copy *SKN7* weakly suppresses the temperature-sensitive growth phenotype of this strain. + indicates growth at 35.5°C.

Column E shows expression in cells of the *cdc42-1* mutant (kindly provided by John Pringle) which do not grow at 37°C but will grow at 35.5°C. High copy *SKN7* antagonises this effect and prevents growth at 35.5°C (N.B. and L.H.J.

5 unpublished observations). + indicates growth at 35.5°C; - indicates no growth at 35.5°C.

Column F shows expression in *skn7D* cells which are acutely sensitive to H₂O₂ (44). + indicates suppression of sensitivity.

10 Column G shows expression in *pkc1-8*, which is a temperature sensitive *pkc1* mutant that grows poorly at 37°C (37) but is lethal in combination with *skn7D* (32,33). Single copy *SKN7* derivatives were expressed and scored for growth at 37°C (+).

15 Column H shows expression in *swi4ts swi6D* cells which are temperature-sensitive for growth at 37°C but can be suppressed by high copy expression of *MBP1*, provided functional *SKN7* is present (N.B. and L.H.J., manuscript in preparation). High copy *SKN7* derivatives were scored for
20 their ability to permit growth of a *skn7 swi4ts swi6D* strain at 37°C. + indicates growth at 37°C.

The *Skn7D427N* and *Skn7Δ(353-623)* mutants behaved exactly as expected in all the assays (Table 3, rows 2,4). The *Skn7ΔHR* protein was active in one of the assays tested,
25 preventing growth of *cdc42* at 35.5°C (Table 3 column E). This result demonstrates that the ROCK-I/Kinectin homology region is not required for this aspect of *Skn7* function, and serves as a positive control since it confirms the structural integrity of the *Skn7ΔHR* protein. In sharp contrast, the
30 *Skn7ΔHR* mutant was inactive in all the other assays examined (Table 3). Thus, the ROCK-I/Kinectin homology region is essential for the bulk of *Skn7* function *in vivo* as well as its interaction with Rho1 and RhoA.

35 Discussion

In this work we used a two-hybrid screen to identify potential effector proteins of the mammalian Rho-family

GTPase RhoA. We identified cDNAs encoding two previously characterised effectors, ROCK-I and Kinectin, together with cDNAs for mNET, the mouse homolog of NET (40), a putative guanine nucleotide exchange factor (GEF), and a novel protein, mDia2, which is related to p140 mDia (17). Our Kinectin cDNA spans codons 1053-1327, a region is distinct from that identified as a RhoA binding domain in a previous two-hybrid screen (15), which suggests that the protein contains multiple Rho-binding elements. Although the ROCK-I, Kinectin and mDia2 proteins bound to both RhoA and Cdc42 in a GTP-dependent manner, mNET exhibited similar affinities for both GTP- and GDP-bound RhoA. This behaviour was not unexpected because the protein contains a Dbp-homology domain, associated with Rho-family guanine nucleotide exchange-factor activity (for review see 45); indeed, we have found that mNET acts as a RhoA GEF both *in vitro* and *in vivo*. In addition to significant sequence homology between mDia2 and p140mDia within the RhoA binding domain, mDia2 also contains a region homologous to the p140 mDia formin homology domain; characterisation of the mDia2 protein is in progress.

Our data implicate ROCK-I, Kinectin and mDia2 as effectors for both RhoA and Cdc42. However, although previous studies of ROCK-I using overlay and two-hybrid assays broadly concur concerning its interactions with RhoA, its ability to interact with other Rho-family proteins has been contentious. Two studies reported weak interaction between ROCK-I and activated Cdc42, although one observed no interaction with wildtype Cdc42 (46,47); interactions with activated Rac1 have also been reported (47,48). We are confident that the interaction between wildtype Cdc42 and ROCK-I detected by our assays is specific, because specificity controls with the interaction domains of PAK65, an MSE55-related protein, and PKN clearly demonstrate Cdc42/Rac-specific and RhoA-specific interactions by the CRIB and REM-1 interaction domains. We have also observed interaction between GTPase binding fragments of ROCK-I and

activated Cdc42.V12 in microinjection assays in mammalian cells. The discrepancy between our data and those obtained by others using overlay assays may reflect the stringency of the overlay assay compared with the two-hybrid and affinity chromatography approaches used here.

Sequence comparison of the Kinectin cDNA recovered from the two-hybrid assay with the minimal RhoA binding domain of ROCK-I (5,43) revealed a 20 amino acid homology between the two proteins. This sequence, which is unrelated to that of the mDia2 Rho-interaction domain, is both necessary for interaction with GTP-bound RhoA and Cdc42 and can by itself can interact weakly with these GTPases. Intriguingly, the distinct Kinectin cDNA previously isolated as a potential RhoA effector also contains a sequence related to the ROCK-I/Kinectin homology region (15). The ROCK-I/Kinectin homology occurs within a region of predicted extended coiled-coil structure, and the REM-1 motif that mediates interactions with PKN/PRK1, Rhotekin and Rhophilin (14) also may have helical character. Sequences related to the ROCK-I/Kinectin homology are present in heterotrimeric G protein β subunits and in the yeast two-component protein Skn7, also within putative coiled-coil regions. However, although we could demonstrate that both β 2 and Skn7 interact with GTP-bound RhoA and Cdc42 and that the interaction is dependent on the ROCK/Kinectin homology, the strongly helical character of the sequence has precluded its use as a search string in database searches for further Rho-interacting proteins.

The ability of the isolated ROCK-I/Kinectin homology to bind RhoA weakly in vitro suggests that it makes direct contacts with the GTPase. However, previous studies of ROCK-I have shown that point mutations or deletion of sequences outside the ROCK-I/Kinectin homology also reduce RhoA binding in both two-hybrid and overlay assays, although the precise effects vary, presumably owing to the different assay conditions used (5,43). In agreement with a previous study (5) we found that mutation of sequences highly

conserved between ROCK-I and ROCK-II substantially reduced interaction with RhoA in both our assays. These sequences might act to stabilise the secondary structure of the ROCK-I/Kinectin homology; alternatively, they might represent a second GTPase docking site. Further studies will be necessary to resolve this issue.

The significance of the potential interaction of Rho-family GTPases with heterotrimeric G protein β subunits remains unclear. Our results are in agreement with a previous report indicated that $G\beta\gamma$ can bind both RhoA and Rac1, and may be involved in membrane targeting of these proteins (49). The putative Rho-interaction surface at the β subunit N-terminus has also been implicated in other protein interactions such as binding of the Ste20 kinase and Cdc24 GEF in *S. Cerevisiae* (50,51). It will be necessary to examine the behaviour of appropriate point mutants in suitable functional assays to assess the significance of these interactions.

Our results show that yeast Skn7 can interact with both yeast Rho1 and its mammalian homolog RhoA, and that the ROCK-I/Kinectin homology region is required for this interaction. The ROCK-I/Kinectin homology is also required for the biological activity of Skn7 *in vivo*: in a number of assays for Skn7 function, deletion of this region inactivated the protein. *SKN7* functions in the oxidative stress response (44) and in G1 cyclin synthesis (33), but its mechanism of action is not yet understood. Several observations suggest that it also plays a role in cell morphogenesis, possibly at the level of cell wall synthesis (31-33). In particular, *skn7 pkc1* double mutants are inviable owing to massive lysis at the small-budded stage of the cell cycle (32,33), a phenotype highly reminiscent of *rho1* mutants (22). These observations suggest that Skn7, like Pkc1, might be a Rho1 effector and, consistent with this notion, high level overexpression of Skn7 is lethal owing to weakening of the cell wall (33), as would be expected if Skn7 were titrating Rho1. Our demonstration that Rho1 and Skn7 can physically

interact, and that the region of the protein that mediates the interaction is required for Skn7 function *in vivo*, provides strong support for the idea that Skn7 is a Rho1 effector. Consistent with this, a multicopy plasmid
5 expressing Rho1 partly suppresses the lethality induced by Skn7 overexpression.

In summary, we have identified a new Diaphanous-related protein and the putative exchange factor mNET as targets of RhoA and Cdc42. A region of similarity between the RhoA
10 binding domains of ROCK-I and Kinectin was used to identify related sequences in yeast Skn7 and the heterotrimeric G protein subunit $\beta 2$. These proteins were also shown to interact with RhoA and Cdc42, and studies of Skn7 indicates that these interactions are functionally significant *in vivo*.

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Table 1

cDNAs recovered in two-hybrid screen

cDNA	Identity ^a	Codons ^b
D1 D4 D9	mouse ROCK-I (U58512)	300-1030 456-1028 349-1025
D2 D3	mouse Kinectin (L43326)	1053-1327
D5	mouse NET1 Dbl-domain bearing Rho-family GEF 82% identical to human NET1 (U02081)	1-595
D7	mDia2 (N-terminal EST: AA415402) related to p140 mDia (U96963)	47-800 ^c

Table 2

Fusion protein	His3 activity			—
	RhoA.V14	Rho1p.V19	Cdc42.V12	
1 ROCK-I (348-1018) (D9)	4	4	4	
2 ROCK-I (831-1010)	3	nt	3	
3 ROCK-I (831-1010) Δ HR	-	-	1	
4 ROCK-I (831-1010)TT	1	nt	2	
5 ROCK-I (950-972)	2	nt	2	
6 Kin (1053-1327)	4	1	4	
7 Kin (1053-1327) Δ HR	-	-	1	
8 SKN7	2	2	3	
9 SKN7 Δ HR	-	-	-	
10 β -2	3	2	3	
11 β -2 Δ HR	-	-	1	
12 β -2 (1-32)	2	2	3	
13 Vector	-	-	1	

Table 3

The ROCK/Kinectin homology is required for SKN7 function

	A	B	C	D	E	F	G	H
aSkn7 protein	pGAL overexpression	swi6Δ p3MCB-lacZ	swi4ts swi6Δ	RhoA	cdc42	H ₂ O ₂	pkc1-8 skn7Δ	swi4ts swi6Δ skn7Δ Yep MBP1
1 SKN7	lethal	+++	+	+	-	+	+	+
2 skn7D427N	lethal	+++	-	nd	-	+	-	+
3 skn7ΔHR	viable	+/-	-	-	-	-	-	-
4 skn7Δ353-623	nd	-	-	-	+	-	-	nd

CLAIMS:

1. An assay for a putative modulator of cell growth comprising:
 - a) bringing into contact a Skn7 polypeptide, a Rho polypeptide and a putative modulator compound under conditions where the Skn7 polypeptide, in the absence of modulator, is capable of binding to the Rho polypeptide; and
 - b) measuring the degree of modulation of binding between the Skn7 and Rho polypeptides caused by said modulator compound.
2. An assay for a putative modulator of cell growth comprising:
 - a) bringing into contact a $\beta 2$ polypeptide, a Rho polypeptide and a putative modulator compound under conditions where the $\beta 2$ polypeptide, in the absence of modulator, is capable of binding to the Rho polypeptide; and
 - b) measuring the degree of modulation of binding between the $\beta 2$ and Rho polypeptides caused by said modulator compound.
3. An assay for a putative modulator of cell growth comprising:
 - a) bringing into contact a polypeptide consisting essentially of a ROCK/Kinectin homology domain, a Rho polypeptide and a putative modulator compound under conditions where the homology domain polypeptide, in the absence of modulator, is capable of binding to the Rho polypeptide; and
 - b) measuring the degree of modulation of binding between the homology domain and Rho polypeptides caused by said modulator compound.
4. A polypeptide consisting essentially of from 8 to 40

amino acids which includes the following sequence:

$X^1X^2X^3X^4LX^6X^7X^8X^9X^{10}X^{11}LX^{13}X^{14}X^{15}X^{16}X^{17}X^{18}X^{19}X^{20}X^{21}X^{22}$

wherein:

X^1 is D, E, A or T;

X^2 is ϕ where ϕ is a hydrophobic amino acid;

X^3 is X where X is any amino acid;

X^4 is N, Q, M or A;

X^6 is R, A or K;

X^7 is X;

X^8 is E or R;

X^9 is X;

X^{10} is D or E;

X^{11} is X;

X^{13} is N, Q, E, R or K;

X^{14} is E, M, N or K

X^{15} is E, Q, R or K

X^{16} is ϕ ;

X^{17} is X;

X^{18} is X;

X^{19} is A or S;

X^{20} is E, Q, R or K;

X^{21} is X; and

X^{22} is E or Q;

provided that when the polypeptide comprises part of the sequence, it comprises the polypeptide of the formula $LX^6X^7X^8X^9X^{10}X^{11}L$ as defined above.

1/2

Figure 1

mDia2	1	MERHRARALGRDSKSSRRKK	GLQSA	PPA	GPYEPGEKRRPKLHL	NI	RT	LT	DD	MLDK	*																																							
p140mDia	1	MEPS	GGGLG	PGR	CTRD	KKKGR	S	PDEL	PAT	CGDGGKHKK																																								
Dm Dia	1					MSRHE	KT	KS	TG	CGGLDS																																								
mDia2	54	FASI	--RI	PGSKKER	PP	LPH	LK	TV	SG	IS	DS	SS	LS	SE	TM	EN	NP	KA	LP	ES	EV	LK	L																											
p140mDia	39	ELER	--FT	SMRIKK	KEKEN	--	SA	HR	NS	SS	AS	YG	DD	PT	AQ	SL	QD	IS	DE	QV	LV	L																												
Dm Dia	18	LFGR	PS	SK	SG	GT	ISS	GT	L	AH	GR	PV	S	AD	NY	V	P	G	VE	DF	EQ	YI	QQ	LS	V	A	E	L	D	A	K																			
mDia2	105	FEKMM	ED	NN	LN	ED	KK	A	PL	RE	KD	F	G	IK	K	EM	V	Q	Y	I	N	T	A	SK	T	G	SL	RS	SR	Q	I	S	P	Q	E															
p140mDia	87	FEQML	V	DM	N	NE	E	K	Q	P	LR	E	K	D	I	V	IR	EH	V	S	O	Y	L	H	T	-	S	K	A	C	M	N	Q	K	E	S	S	R	S	A	M									
Dm Dia	71	ELEI	I	ED	NI	P	K	D	K	R	E	P	L	A	K	S	K	E	E	R	Q	K	M	I	M	W	H	L	K	G	N	S	L	E	R	S	A	N	S	R	F	E	K	P	I	D				
mDia2	158	FLHEL	K	M	G	Y	T	D	E	R	-	L	F	T	Y	L	E	S	L	R	V	S	L	T	S	H	P	V	S	W	V	Q	S	E	G	H	E	G	L	G	L	L	D	I	L	E	K	L	I	
p140mDia	139	YIQEL	R	S	C	L	R	D	M	H	-	L	L	S	C	L	E	S	L	R	V	S	L	N	N	P	V	S	W	V	Q	T	F	G	A	E	G	L	A	S	L	L	D	I	L	K	R	L	H	
Dm Dia	124	YVEY	H	Q	N	G	E	H	S	T	H	K	V	Y	Q	C	V	E	S	L	R	V	A	L	T	S	N	P	I	S	M	I	K	E	F	G	V	A	G	I	G	T	I	E	K	L	A	R	S	K
mDia2	210	N	--GQ	I	Q	E	X	V	V	K	T	Q	H	K	V	I	Q	C	L	R	A	L	M	N	T	Q	Y	G	L	E	R	I	M	S	D	K	R	S	--L	S	L	L	A	K	A	N	D			
p140mDia	191	D	E	K	E	E	T	S	G	N	Y	D	S	R	N	O	H	E	I	R	C	L	K	A	T	M	N	N	K	F	G	I	K	T	M	L	E	T	E	G	--I	L	L	L	V	R	A	N	D	
Dm Dia	177	NN	A	S	Y	--	--	--	--	--	E	K	I	E	F	E	A	I	R	C	L	K	A	I	M	N	N	T	W	G	L	N	V	L	N	P	D	Q	H	S	V	V	L	L	A	Q	S	L	D	

Figure 2

A

mKinectin	1177	SFTASERELERLRQEN...KDMENLRRIEREHHEMELEKAEEMERSTYVMEVRELKDLLTELQ
mROCK-I	930	EITDKDHTVSRLEETNSVLTKDIEMLARKENEEIANERMRTAEEYKLLKKEEIN..NLKAAFE
mROCK-II	950	ELTEKDTTASLEETNRTLTSDEVANLANEKEELNNKLKDSOEQLSKLKDEMSAAAIAKAOFE
mKinectin	1135	KKLDDSYSEAVRQNEELNLLKTQLNETHS.KLQNEQTERKKVADDLHKAQQSLNSIHSKISL
mROCK-I	990	KNISTERTLKTOAVNFAEIMNRKDFKIDRKKANTQDLRKKKEKENRKL.QLELNQEREKFNQ
mROCK-II	1012	KOLLNERTLKTOAVNKLAEIMNRKE...PVKRGSDTDVRRKEKENRKL.HMELKSEREKLTQ

B

mKinectin	1192	NKDEMELRLRERERHLEMELEKAEEME	1115
mROCK-I	949	TKDETMLRKENEELNEERMRTAEFE	972
mROCK-II	893	TSDEVALANEKEELNFKDKSQEQ	916
β2	1	MSDEQLRQEAQLRNEQTRDAIRKA	23
Skn7	237	KDAEGMLRRRVDEKLQKEADMSKHE	260
mKinectin	831	EKTQVALAKQEIENVLKELGNAQLE	854



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(21) International Application Number: PCT/GB99/01096 (22) International Filing Date: 9 April 1999 (09.04.99) (30) Priority Data: 9807848.8 9 April 1998 (09.04.98) GB (71) Applicants (for all designated States except US): MEDICAL RESEARCH COUNCIL [GB/GB]; 20 Park Crescent, London W1N 4AL (GB). IMPERIAL CANCER RESEARCH TECHNOLOGY LIMITED [GB/GB]; Sardinia House, Sardinia Street, London WC2A 3NL (GB). (72) Inventors; and (75) Inventors/Applicants (for US only): JOHNSTON, Leland, Herries [GB/GB]; Division of Yeast Genetics, National Institute of Medical Research, The Ridgeway, Mill Hill, London NW7 1AA (GB). TREISMAN, Richard, Henry [GB/GB]; Imperial Cancer Research Fund Laboratories, 44 Lincoln's Inn Fields, London WC2A 3PX (GB). (74) Agents: BRASNETT, Adrian, H. et al.; Mewburn Ellis, York House, 23 Kingsway, London WC2B 6HP (GB).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> (88) Date of publication of the international search report: 2 December 1999 (02.12.99)
(54) Title: POLYPEPTIDE DOMAINS CAPABLE OF BINDING RHO PROTEIN AND THEIR USE IN ASSAYS		
(57) Abstract <p>The Rho family of GTPases are involved in a wide range of eukaryotic cellular processes. Several known effector proteins bind to these GTPases and mediate their effect. Such proteins include ROCK-I/p160ROCK, ROCK II and Kinectin. The present invention provides a novel twenty three amino acid Rho-binding region which is conserved in a range of Rho binding proteins. Other proteins, including the yeast protein Skn7, are shown to contain regions homologous to this conserved region and to interact with Rho GTPases. Assays are also provided to identify molecules which affect cell growth through modulation of the Rho GTPase/effector protein interaction.</p>		

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INTERNATIONAL SEARCH REPORT

International Application No

PL./GB 99/01096

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C07K14/47 G01N33/573 C12Q1/48 G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 05249 A (THE GOVERNMENT OF THE UNITED STATES OF AMERICA) 13 February 1997 (1997-02-13) seq id no 34, 38 ---	4
X	FLEISCHMANN ET AL.: "Hypothetical protein HI1717" SWISSPROT SEQUENCE DATA BASE, 1 November 1995 (1995-11-01), XP002118148 Ac P44295 the whole document ---	4
A	WO 95 21252 A (UNIV LELAND STANFORD JUNIOR) 10 August 1995 (1995-08-10) seq id no 42 page 3, line 9 - line 37 --- -/-	1-4

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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INTERNATIONAL SEARCH REPORT

International Application No

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>LEUNG ET AL.: "Kinectin 1 (coiled-coil protein)" EMBL SEQUENCE DATABASE, 1 November 1996 (1996-11-01), XP002118149 HEIDELBERG DE Ac Q61595 the whole document & SKINNER ET AL.: "Cloning of novel kinectin splice variants with alternative C-termini: structure, distribution and evolution of mouse kinectin" IMMUNOL CELL BIOL, vol. 74, 1996, pages 421-433,</p>	4
A	<p>NAKAGAWA ET AL.: "Rho-associated, coil-coil forming protein kinase P160 ROCK-1" EMBL SEQUENCE DATABASE, 1 February 1997 (1997-02-01), XP002118150 HEIDELBERG DE Ac P70335 the whole document & NAKAGAWA ET AL.: "ROCK-I and ROCK-II, two isoforms of Rho-associated coiled-coil forming protein serine/threonine kinase in mice" FEBS LETT, vol. 392, 1996, pages 189-193,</p>	4
A	<p>NAKAGAWA ET AL.: "Rho-associated, coiled-coil forming protein kinase P160 ROCK-2" EMBL SEQUENCE DATABASE, 1 February 1997 (1997-02-01), XP002118151 HEIDELBERG DE Ac P70336 the whole document & NAKAGAWA ET AL.: "ROCK-I and ROCK-II, two isoforms of Rho-associated coiled-coil forming protein serine/threonine kinase in mice" FEBS LETT, vol. 392, 1996, pages 189-193,</p> <p style="text-align: center;">--- -/--</p>	4

INTERNATIONAL SEARCH REPORT

International Application No

PL./GB 99/01096

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BROWN ET AL.: "Putative transcription factor SKN7 (POS9 protein)" SWISSPROT SEQUENCE DATA BASE, 1 February 1995 (1995-02-01), XP002118152 Ac P38889; P39747 the whole document -& BROWN ET AL.: "Yeast Skn7p functions in a eukaryotic two-component regulatory pathway" EMBO J, vol. 13, 1994, pages 5186-5194, XP002069763 ---	4
P,Y	WO 98 20127 A (ONYX PHARMA INC) 14 May 1998 (1998-05-14) cited in the application abstract; claims 50,51 page 2, line 13 - line 29 ---	1-3
P,Y	WO 98 38331 A (JOHNSTON LELAND HERRIES ;MEDICAL RES COUNCIL (GB); RAITT DESMOND C) 3 September 1998 (1998-09-03) cited in the application abstract; claims 7-9 -----	1-3

INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No

PCT/GB 99/01096

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9705249 A	13-02-1997	AU 6842396 A CA 2228374 A EP 0932671 A	26-02-1997 13-02-1997 04-08-1999
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WO 9838331 A	03-09-1998	AU 6629298 A	18-09-1998



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(21) International Application Number: PCT/GB99/01096 (22) International Filing Date: 9 April 1999 (09.04.99) (30) Priority Data: 9807848.8 9 April 1998 (09.04.98) GB (71) Applicants (for all designated States except US): MEDICAL RESEARCH COUNCIL [GB/GB]; 20 Park Crescent, London W1N 4AL (GB). IMPERIAL CANCER RESEARCH TECHNOLOGY LIMITED [GB/GB]; Sardinia House, Sardinia Street, London WC2A 3NL (GB). (72) Inventors; and (75) Inventors/Applicants (for US only): JOHNSTON, Leland, Herries [GB/GB]; Division of Yeast Genetics, National Institute of Medical Research, The Ridgeway, Mill Hill, London NW7 1AA (GB). TREISMAN, Richard, Henry [GB/GB]; Imperial Cancer Research Fund Laboratories, 44 Lincoln's Inn Fields, London WC2A 3PX (GB). (74) Agents: BRASNETT, Adrian, H. et al.; Mewburn Ellis, York House, 23 Kingsway, London WC2B 6HP (GB).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>With amended claims.</i> (88) Date of publication of the international search report: 2 December 1999 (02.12.99) Date of publication of the amended claims: 6 January 2000 (06.01.00)
(54) Title: POLYPEPTIDE DOMAINS CAPABLE OF BINDING RHO PROTEIN AND THEIR USE IN ASSAYS		
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AMENDED CLAIMS

[received by the International Bureau on 8 November 1999 (08.11.99);
new claims 5 and 6 added; remaining claims unchanged (2 pages)]

amino acids which includes the following sequence:

$X^1X^2X^3X^4LX^6X^7X^8X^9X^{10}X^{11}LX^{13}X^{14}X^{15}X^{16}X^{17}X^{18}X^{19}X^{20}X^{21}X^{22}$

wherein:

X^1 is D, E, A or T;

X^2 is ϕ where ϕ is a hydrophobic amino acid;

X^3 is X where X is any amino acid;

X^4 is N, Q, M or A;

X^6 is R, A or K;

X^7 is X;

X^8 is E or R;

X^9 is X;

X^{10} is D or E;

X^{11} is X;

X^{13} is N, Q, E, R or K;

X^{14} is E, M, N or K

X^{15} is E, Q, R or K

X^{16} is ϕ ;

X^{17} is X;

X^{18} is X;

X^{19} is A or S;

X^{20} is E, Q, R or K;

X^{21} is X; and

X^{22} is E or Q;

provided that when the polypeptide comprises part of the sequence, it comprises the polypeptide of the formula $LX^6X^7X^8X^9X^{10}X^{11}L$ as defined above.

5. A polypeptide according to claim 4 wherein:

X^1 is D, E, A or T;

X^2 is ϕ where ϕ is a hydrophobic amino acid;

X^3 is X;

X^4 is N or Q;

X^6 is R;

X^7 is R;

X^8 is R;

X^9 is X;

X^{10} is D or E;

X¹¹ is X;
X¹³ is N or Q;
X¹⁴ is K;
X¹⁵ is E or Q;
X¹⁶ is ϕ ;
X¹⁷ is X;
X¹⁸ is X;
X¹⁹ is S;
X²⁰ is K;
X²¹ is X; and
X²² is E.

6. A polypeptide according to claim 4 or 5 consisting essentially of from 15 to 40 amino acids.

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